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PREFACE

An introduction to the present volume of the *Review* can refer to very few matters that have not received mention in the earlier volumes. Each year, however, it is a pleasure to recall those phases of our editorial duties that bring us into association with the contributors of the reviews and with some of the readers. The cooperation of our colleagues, year by year, in preparing these reviews has been most cordial and complete, despite the vexatious restrictions and importunities with which we perennially tax their patience. Every investigator in biochemistry, impressed as he must be by the fertility of research and the wealth of literature in this expanding science, cannot but be appreciative of the difficulties that attend the labors of a reviewer—the selection of papers for review, their critical interpretation, and their integration into a coherent chapter.

It has also been satisfying and helpful to receive from time to time from the readers of the *Review* suggestions with respect to authorship and subject matter. It is our hope that an increasing number of our colleagues may extend to us the assistance that comes from such suggestions in order that the quality of the *Review* and its usefulness may steadily be enhanced.

In continuation of the policy of including a few reviews on topics of a timely nature, we are glad to publish in the present volume a review by F. B. LaForge on "Organic Insecticides." We regret that the reviews on "Mineral Nutrition of Plants" by D. Sabinin, on "Nitrogenous Constituents of Green Plants" by K. Mothes, and on "Chemotherapy" by E. Fourneau, announced in the fall circular, were prevented by unforeseen circumstances in arriving for publication.

We are happy to report the appointment of Professor Carl Noller of Stanford University as associate editor. In particular, we are grateful to him for editing many of the manuscripts in the present volume and preparing the subject index.

It is also fitting to mention at the present time the forthcoming publication of the *Annual Review of Physiology*—a companion volume to the *Annual Review of Biochemistry*. The new publication will appear as a joint undertaking with the American Physiological Society. It is the confident hope of all associated with the enterprise that the two Reviews may be mutually supplementary and of sub-

stantial value to those engaged in the biological, chemical, and medical sciences. To minimize the possibility of duplication in subject matter, it will be our policy to refrain from including in future volumes any topics that might appear more appropriately in the *Annual Review of Physiology*.

Finally, it has been deemed advisable to change the name of the Corporation, which, as a non-profit agency, will be responsible for the sale of both Reviews. The name is now changed from "Annual Review of Biochemistry, Ltd." to "Annual Reviews, Inc."

We wish to express our thanks to the Stanford University Press for the excellence of its work and its fine cooperation throughout.

C. L. A.
D. R. H.
J. M. L.
C. L. A. S.

ERRATA

Volume IV, page 376, lines 19 and 20: *for* 34 (1934), *read* 24 (1932).

Volume VI, page 202, line 9: *for* *p*-bromophenylmercapturic acid, *read* *p*-bromophenylmercaptan.

Page 277, fourth line from bottom: *for* *l*(+), *read* *d*(-).

Page 404, line 10: *for* pyurvic, *read* pyruvic.

Page 471, line 11: *for* Jolin, *read* Johlin.

Page 486, line 3: *for* Jolin, *read* Johlin.

Page 655: *for* Jolin, *read* Johlin.

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BIOLOGICAL OXIDATIONS AND REDUCTIONS*

By L. MICHAELIS AND C. V. SMYTHE

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New York City*

Reversible oxidation-reduction systems.—The problem of the reversible oxidation of ascorbic acid has been investigated by several authors. Borsook, Davenport, *et al.* claim that there are two stages in the oxidation of ascorbic acid, the first being the formation of the dehydro compound, and the second a non-oxidative rearrangement followed by a further oxidation. Only the first stage is reversible. Ascorbic acid remains much longer in the reduced state in whole blood than in plasma. There is no mechanism in blood for reducing dehydroascorbic acid or retarding the irreversible rearrangement, but in tissues dehydroascorbic acid is rapidly reduced. The principal reducing agent seems to be glutathione. Ghosh & Rama Char have used a special technic for the measurement of the potential in ascorbic acid plus dehydroascorbic acid. The situation is presented in some respects in a simpler way by Ball (1). He finds that the ascorbic acid system is able to establish definite potentials at the electrode only if an intermediary such as thionine, or an iron or copper salt, is used. In the presence of one of these, ascorbic acid can be titrated and behaves as a regular bivalent reversible system at any pH less than 5. At higher pH, the oxidized form becomes unstable.

Bezssonoff & Woloszyn describe the existence of a substance intermediate between ascorbic acid and dehydroascorbic acid. They attribute to it, in their formula, the structure of a bimolecular peroxide, but designate it in the text as an ether. Their evidence is not convincing. Mayer has measured the oxidation potential of reductinic acid. This is much more positive than the potential of ascorbic acid and of reductone.

In the later sections oxidation-reduction potentials are given for the sulfhydryl-disulfide system and for three hydroxy acid-keto acid systems. For the last three, although shown to be reversible, no complete titration curve has been obtained to permit the satisfactory interpretation of the systems.

Uchimura found nearly reproducible potentials at an indifferent

* Received January 17, 1938.

electrode inserted directly in the quadriceps. The value was about $+0.230$ volt. After illumination with ultraviolet light or X-rays there was a reversible drop of 24 to 32 mv. Tang & Lin (1), using a suspension of *Chlorella*, found the oxidation-reduction potential to be more positive in the dark than in the light. The effect was reversible, but not strictly reproducible. Janicki found that the oxidation-reduction potential of aqueous extracts of germinating barley decreases during the germination. This decrease parallels the increase in amylase activity.

In an attempt to measure the limiting value of the reduction potential in suspensions of yeast, Fromageot & Bost found that this value depends very largely on the indicator dye used. The lowest value attainable is that with indigotrisulfonate as indicator and is $rH = 4.2$ at pH 6.4. Wurmser & Filitti-Wurmser have demonstrated the poisoning effect of yellow enzyme in yeast extract. It establishes a plateau of the potential at -0.07 volt.

Some attempts have been made to utilize Heyrowsky's polarograph. Brdička & Tropp showed that the electrolytic reduction of oxygen dissolved in water at the dropping mercury electrode registers two levels of oxidation, obviously corresponding to hydrogen peroxide and water. These are separated not by thermodynamic conditions, but by some condition similar to overvoltage. Addition of hemoglobin or related compounds makes the break in the curve disappear. This latter fact was studied further by Haurowitz *et al.* and will be reported in a later section of this review. The same apparatus was used by Müller & Baumberger for the determination of normal potentials of reversible systems, including those with intermediate steps, as an alternative technic to the customary potentiometric titration.

A number of reversible two-step systems have been added to those known before. A close analogy to the two-step reduction of pyocyanine was found by Preisler & Hempelmann in β -hydroxyphenazine and N-methyl- β -oxyphenazine, two dyestuffs of a structure related to pyocyanine. Hill found another case of two-step reduction in the yellow pigment of the tubercle bacillus, 2-methyl-3-hydroxy-1,4-naphthoquinone (phthiocol). Since this is an anionic dyestuff, the separation of the two steps becomes manifest only in alkaline solution and is noticeable only at a pH greater than 9. The corresponding change of the slope of the titration curve in alkaline solution was not observed in the previous work by Ball (2) but is now confirmed by him (3). Sarver & Kolthoff found a representative of a benzidine compound, diphenyl-

benzidinesulfonic acid, stable enough for titration. The intermediate compound is shown to be a free radical.

Michaelis & Schubert found in phenanthrenequinone-3-sulfonate a dyestuff which is, for various reasons, an especially suitable model for the study of the two-step oxidation of anionic dyestuffs. It shows a crossing point of the three normal potentials lying at a conveniently available pH ($= 10.0$). The greatest separation of the two steps is reached at pH 12 and remains constant from there on. The titration curves, though very much steeper than in a system without an intermediate step, show no jump in the middle part that would indicate the separation of the two steps. This phenomenon caused the authors to develop the theory of the titration curve with respect to the points of inflection. The result is that no jump in the middle part of the titration curve can occur unless the semiquinone-formation constant is greater than 16.

The same dyestuff, due to its high solubility, was employed by Michaelis & Fletcher (2) for the solution of the problem of whether two molecules of a water-soluble semiquinone radical can associate to form a valence-saturated dimeric molecule in the way that many other organic radicals, known to exist in non-aqueous solutions, do. The analysis of the titration curves for various concentrations of the dye revealed the following results: In alkaline solution (pH 12.2) the intermediate form was practically entirely in the radical form if the concentration of the dye was low; at higher concentration there was present, in equilibrium with the radical, the dimeric form; in acid solution (pH $= 4.6$) the radical was capable of existence only to an extremely slight extent, which was difficultly measurable due to lack of resonance; at low concentrations the dimeric form also scarcely existed but it increased rapidly as the total concentration increased.

A semiquinone radical has an odd number of electrons and is expected to be paramagnetic. This is confirmed by measurement of the magnetic susceptibility. In order to obtain results comparable to those obtained with the potentiometric method, the magnetic measurements should be carried out in aqueous solution. This is especially desirable because the skepticism which the asserted existence of the semiquinone radicals aroused in the beginning was mainly due to the fact that no radicals had previously been known to exist as stable molecules in aqueous solution. Michaelis, with Boeker & Reber and with Reber & Kuck, used a method in which a solution of phenanthrenequinone-3-sulfonate was reduced by a slowly acting reducing agent,

glucose in alkaline solution, or methylglyoxal with potassium cyanide in acid solution. During the reduction the magnetic susceptibility of the solution was measured repeatedly. In an alkaline solution a paramagnetic molecular species arises and disappears again when the reduction is complete. In acid solution no radical was formed. This is in agreement with the potentiometric result just described.

Just as all dyestuffs resembling phenazine, lactoflavin (vitamin B₂) and the other flavin dyes are capable of forming an intermediate step of oxidation-reduction. This had been shown by Kuhn & Wagner-Jauregg for extremely acid solutions, where the intermediate form is of red color. Michaelis, Schubert & Smythe showed that to a slight extent (10 per cent of the total dye *in maximo*) the intermediate form also exists at higher pH values (2 to 12) and then is green in color. Kuhn & Ströbele showed that in the crystalline, solid state not only this green form exists, but also two others. They prepared: (a) verdoflavin, on an oxidation level corresponding to one flavin with one monohydroflavin radical and having a paramagnetic susceptibility as though half of it were in the radical form; (b) rhodoflavin, consisting of one dihydroflavin with one monohydroflavin radical, both as hydrochlorides; and (c) chloroflavin, a compound intermediate between *a* and *b*, of the composition of the free radical, but of a much too low paramagnetic susceptibility. Michaelis & Schwarzenbach showed that among all these possible intermediate forms only the free radical is capable of existence in dilute aqueous solutions of lactoflavin. In solutions of higher concentration such as never occur physiologically, a dimerized form exists in equilibrium with the radical, but none of the other forms is present. The formation of an intermediate step of oxidation-reduction in solutions of the flavin dyestuffs was confirmed also by Klemperer *et al.* using the index potential as evidence.

Haas describes the important observation that when the yellow respiration enzyme (lactoflavinphosphate ester with a specific protein) is being reduced at 0° C by the reduced form of the coenzyme (triphosphopyridine nucleotide) an intermediate red color appears. This red compound has the same absorption spectrum as the red radical that appears when free flavin is reduced at a pH less than 0. From this result the combination with the specific protein¹ and coenzyme in neutral solutions seems to have the same effect as has a strong acid,

¹ This combination of flavinphosphate with the specific protein has been studied during the year by Theorell (2).

i.e., the equilibrium is displaced in favor of the radical. Lipmann (1) observed that vitamin B₁ (thiamine), on reduction with sodium hydro-sulphite or with zinc dust in acid solution, also shows an intermediate form of reduction having a green color.

The idea that the formation of an intermediate radical in organic dyestuffs should be linked in some way or other with oxidation processes was suggestive, of course, ever since the discovery of these radicals. In this connection Shaffer's theory of what we may call "valence harmony" in oxidation-reduction should be mentioned first. He starts from the observation that certain simple systems react very slowly in spite of a considerable potential difference. Thus Ce^{++++} oxidizes Tl^+ only very slowly and iodine reacts very slowly with Ti^{+++} . These reactions are catalyzed, however, by a manganese salt or by certain dyestuffs. Shaffer suggests that the reason these reactions are slow is that the electron change involved is not the same for the oxidant and the reductant. Thus Ce^{++++} can accept only one electron and forms Ce^{+++} , but Tl^+ must lose two electrons at once and form Tl^{++} . No Tl^{++} compound is known. In order for this reaction to occur a trimolecular collision must take place. To be an effective catalyst in such a reaction a compound must make a trimolecular collision unnecessary. Manganese can do this for it can lose and accept one or two electrons and hence can replace a trimolecular collision with a series of bimolecular collisions. A dyestuff which is capable of semiquinone formation could also act as a catalyst for the same reason. Some of the dyes that Shaffer found to be effective are known to form semiquinones and in some other cases where a semiquinone was not known he showed that it really does exist, e.g., in the indigo dyes.

An idea, similar but not identical with Shaffer's hypothesis of valence harmony, is what may be called the principle of "compulsory univalent oxidation," as discussed by Michaelis and soon thereafter demonstrated by Michaelis & Fetcher (1) in the following model: Benzoin, $C_6H_5CO \cdot CHOH \cdot C_6H_5$ can be easily oxidized to benzil, $C_6H_5CO \cdot CO \cdot C_6H_5$, either by oxygen or by iodine, in alkaline alcoholic solution. It is not oxidized in acid solutions. Under the same alkaline conditions that permit oxidation, an intermediate compound of purple color is formed which is proven to be a free radical. In alkaline solution the hydroxyl group attached to the trivalent carbon of this radical is ionized: $C_6H_5CO \cdot CO^- \cdot C_6H_5$. The ion of this formula is in resonance with another in which the negative charge

is at the other oxygen atom, and since resonance is a stabilizing factor this radical is stable. Resonance can be expected to be especially strong in such cases where the odd electron is shared by two equal atoms (two nitrogen atoms in phenazine compounds and in the flavins; two oxygen atoms in the quinones and in benzil). In this case oxidation proceeds only under the same conditions as permit of formation of the intermediate free radical. In acid solution, where oxidation does not occur, the radical would be non-ionized hence it would have no resonance and so would scarcely be capable of existence.

The following general principle may be postulated: In most organic compounds an oxidation of any valence-saturated compound to another such compound on a higher level of oxidation is a bivalent oxidation. The inertia of organic compounds toward oxidizing agents is due to the fact that the oxidation can proceed at a measurable speed only in two successive univalent steps and consequently only if the intermediate radical can be formed. If the normal potential of the first step of oxidation is much higher than that of the second step the amount of the radical formed may be extremely slight and its concentration may be the limiting factor for the speed of oxidation. In many organic compounds this situation leads to a practical lack of reactivity at ordinary temperatures in absence of a catalyst. The role of an enzyme, then, is to displace the equilibria concerned in favor of the radical. A possible demonstration of this principle is Haas' experiment cited above: the combination with a specific protein and a coenzyme displaces the equilibrium in favor of the radical.

It should be claimed, to make the argument complete, that the coenzyme is also capable of forming an intermediate step of oxidation-reduction. In fact this has been shown to be true by Adler, Hellström & Euler, Karrer, Schwarzenbach, Benz & Solmssen, and Hellström. The evidence is, in part, that there is an intermediate yellow color formed during the reduction and, in part, that spectrophotometrically it can be shown that during the first part of the reduction with hydrosulfite more reductant is used than corresponds to the dihydropyridine which arises. It should, however, be noted that these observations are not sufficient for the identification of the yellow substance.

Bancroft & Magoffin also discuss in a broad critical review the nature of the activation energy in oxidation and come to the conclusion that this is the energy hump which is necessary to form an inter-

mediate step of oxidation-reduction of thermodynamically lower stability.

The dismutation of a free radical has to be clearly distinguished from the type of dismutation known as the Cannizzaro reaction, in which both the oxidation and the reduction are bivalent. Of this type, Levene & Christman have described a new example in which glucosamine in a hydrogen atmosphere with platinum as a catalyst dismutates into aminosorbitol and glucosaminic acid.

Metal complexes in oxidation-reduction.—Great strides have been made in the preparation of pure metal complexes of acknowledged importance for oxidation studies. Sumner & Dounce have developed a simple method of preparing pure catalase in crystalline form from beef liver. They use dioxane and ammonium sulfate for fractionation. The red crystals are a haemin-protein compound. When dissolved in acetone and hydrochloric acid they give rise to a blue color. The iron content is not yet definitely determined but is about 0.11 per cent.

An oxidase from potatoes has been prepared by Kubowitz (1) and is estimated to be 50 per cent pure enzyme. It is the copper compound of a specific protein. The preparation contains no catalase and no peroxidase. Its catalytic behavior in combination with other enzymes will be discussed in a later section of this review. Stotz, Harter & King consider the ascorbic acid oxidase, present in cabbage leaves and other vegetable tissues, to be a protein-copper compound also, but they do not consider the protein to be specific.

Keilin & Hartree (2) have prepared pure cytochrome-*c* from heart muscle by extraction with trichloroacetic acid and fractional precipitation with ammonium sulfate. It has an equivalent weight of 16,500 and a molecular weight (osmotic pressure method) of the same order. The iron content is 0.34 per cent. Its chemical properties are those expected from previous preparations. It cannot form an oxygen compound nor is it oxidized from the ferrous to the ferric state by oxygen. It is oxidized by indophenoloxidase. It is not affected by boiling. Mori *et al.* confirm Keilin's statement that cytochrome-*c* is catalytically oxidized by indophenol oxidase. Certain metal complexes, especially cobalt complexes, e.g. $(\text{Co}, \text{en}_2, \text{Cl}_2)\text{Cl}$, and also pyridine-haemin have the same catalytic effect.² Certain other metal complexes, and oxidases from a fungus, are without effect.

Yakushiji & Mori seem to have succeeded in synthesizing cyto-

² en = ethylene diamine.

chrome-*b*, at least in solution. They claim that a combination of the specific protein of the yellow respiration enzyme with protohaematin yields a haemochromogen spectroscopically indistinguishable from cytochrome-*b*. Katagiri *et al.* report a synthesis of a haemochromogen resembling cytochrome-*c*: They started with protoporphyrin and treated it with sodium hydrosulfite; from the product they isolated what they call porphyrin-*c*; this combined with iron and pyridine to form a compound resembling cytochrome-*c* spectroscopically and chemically.

Barkan & Schales prepared a haemin from mammalian blood which seems to be identical with the haemin of cytochrome-*c*. This "haemin-*c*" amounts to four per cent of the total haemin of blood and is probably combined with globin to form a part of the haemoglobin. It is prepared most easily from a peptic digest of blood. When extracted with ether the ordinary haemin is removed and haemin-*c* remains in the aqueous phase.

Sato & Tamiya have discovered by spectroscopic analysis the presence in living *Paramaecium* of cytochrome-*a* and -*c*, and a haemoglobin with absorption bands very close to, but not quite identical with, those of human haemoglobin. Tamiya & Ogura have studied the role of the various cytochromes in the mechanism of respiration and conclude that their normal potentials decrease in the order *c*, *a*, *b*. Cytochrome-*c* is not autoxidizable but is partially oxidized by cytochrome-*a*, which is autoxidizable. Tamiya & Sato discuss theoretically the state of oxidation-reduction of each of the three cytochromes in a mixture of the three, both anaerobically in a state of thermodynamical equilibrium and aerobically in a kinetically stationary state.

A peculiar spectroscopic observation is reported by Urban & Eaton concerning the complex porphyrin formerly obtained by Coulter from diphtheria toxin and cytochrome-*b*. These two substances behave toward cytochrome-*c* in a way which is interpreted as follows: When the iron atom of cytochrome-*c* is in the bivalent state the two compounds can be oxidized by the porphyrin ring of cytochrome-*c*, but when the iron is in the trivalent state, this ring cannot oxidize those substances.

An important observation by Theorell (1) concerning the mode of attachment of the protein to porphyrin in cytochrome-*c* should also be mentioned, although it is not directly concerned with oxidation-reduction.

Yoshikawa has grown yeast in an iron-free medium and has com-

pared the yield and some of its properties with other yeast grown in the presence of various amounts of added iron and copper. There is a decided influence of iron on growth when added in amounts of from 10 to 1000 $\mu\text{g.}$ of ferrous ammonium sulfate per liter. The effect increases rapidly up to 100 $\mu\text{g.}$ and slowly up to 1000 $\mu\text{g.}$, which is the optimum. The same effect is found in the production of iron-haematin compounds. If, in addition to 100 $\mu\text{g.}$ of iron, copper sulfate is added, there is a great improvement in growth and formation of iron-porphyrin. Ten $\mu\text{g.}$ of copper per liter is the optimum. The strains from these various media showed marked differences in oxygen consumption. The aerobic production of carbon dioxide was decreased in yeasts raised with iron, or with iron plus copper. The anaerobic fermentation, however, was only slightly influenced. Thus the Pasteur effect is greater in the order: iron-copper yeast, iron yeast, metal-free yeast. The catalase content increases considerably in yeast raised with iron. Especially interesting is the fact that the amount of indophenoloxidase increases much more in iron plus copper than in iron alone. This fact is in excellent agreement with Kubowitz' statement, reported above, that oxidase is a copper compound.

The formation of methaemoglobin from haemoglobin during respiration has been studied by Bernheim & Michel in various tissues. They find it to be correlated with the oxygen uptake, and not to be catalytically increased by copper or any indifferent colloidal material. The formation of methaemoglobin during respiration decreases in the order: kidney, liver, brain, muscle. Methaemoglobin is reduced to haemoglobin in tissues only under anaerobic conditions. M. Fischer claims that oxyhaemoglobin forms a complex with ascorbic acid without oxidizing it, and that the unaltered ascorbic acid may be displaced by carbon monoxide or potassium cyanide. Similar results were obtained by Gabbe. Emmerie & Eekelen, however, obtained different results. They find that ascorbic acid is not absorbed by oxyhaemoglobin but is oxidized to the reversible stage. Barnard (1) shows that methaemoglobin, when produced with ferricyanide, combines with hydrogen peroxide to form a complex compound such as described by Keilin & Hartree, but when produced by quinone it reacts with hydrogen peroxide so as to form oxyhaemoglobin. Barnard (2) also studied the reaction of haemoglobin and related compounds with nitrite ion, which may react in two ways: It may combine with the iron atom by an ionic bond or it may react with the molecule attached to the haem in a way, as yet unknown, that is independent of the oxi-

dation level of the iron. In methaemoglobin this reaction amounts to denaturation of the protein. Brooks has studied the same reaction. He finds that sodium nitrite reacts with haemoglobin in the absence of both oxygen and a reducing agent to form one mol of nitric oxide haemoglobin (comparable to carbon monoxide haemoglobin) and one mol of methaemoglobin. In the presence of a reducing agent, haemoglobin is completely converted to nitric oxide haemoglobin.

Millikan has elaborated a photoelectric device by which the degree of oxygen saturation of the intracellular muscle haemoglobin in a cat's muscle can be measured instantaneously and recorded. He shows that the oxygen storage is enough to tide the muscle over from one contraction to the next.

The oxidation-reduction potentials in haemochromogen systems have been studied by Barron (1). The potentials are most reproducible and most quickly established with cyan-haemochromogen, and in the pH range 8.2 to 12.0. Within this range the potential is independent of pH, of the kind of buffer, and of the concentration of the haemochromogen, and the titration curve is that of an ordinary univalent system. In haemochromogens containing a more loosely bound nitrogenous substance the potentials become more dependent on the three variables. Thus, the shape of the titration curve in pyridine-haemochromogen may gradually vary from a one-electron curve at low pH to a two-electron curve at pH 11.98. The situation is perfectly clear only in the cyan compound. In all the others the difficulties arising are attributed to the inclination of the haem-compounds to form molecular aggregates in solution. In another paper Hogness, Zscheile, Sidwell & Barron show by spectroscopic analysis that cyan-haemochromogen in solution is unimolecular, whereas ferrihaem-hydroxide (i.e., haemin in alkaline solution) is dimeric.

Havemann & Wolff reinvestigated the problem of whether haemoglobin plus methaemoglobin form a reversible oxidation-reduction system that can be potentiometrically titrated. They found this to be the case, though the establishment of the potentials was slow. According to their results, $E = +0.526 - RT/F \times \text{pH} + RT/F \log ([\text{Methb}]/[\text{Hb}])$ at 38°. The reviewers find a contradiction in this equation to the author's justifiable assumption that $\text{Hb} - 4\text{e} \rightleftharpoons \text{Methb}$. Such a reaction would require the factor $RT/4F$ instead of RT/F , or else at least an indication of a four-step titration curve.

A model for the potentiometric behavior of a quadrinuclear iron complex was found by Michaelis & Smythe in the pentacyano-aquo

complex of iron, $[\text{Fe}(\text{CN})_5\text{H}_2\text{O}]\text{Na}_x$, where x is either 2 or 3 (ferro or ferri complex). It is shown that this complex in solution is polymerized to a quadrimolecular aggregate and that the four iron atoms during a potentiometric titration are reversibly reduced in four successive steps of which the first overlaps the second, and the third overlaps the fourth, whereas the second and third overlap but little.

Measurements of the paramagnetic susceptibility of haem compounds have been carried out by Pauling & Coryell and Pauling, Whitney & Felsing. From the susceptibility it is inferred that the number of unpaired (magnetically unbalanced) electrons in ferri-haem is 5; in ferro-haem it is 4; in globin-, pyridine-, nicotine-, and dicyanide-haemochromogen, and also in nickel porphyrin, it is 0. From this it is concluded that iron in haemoglobin is attached to the four nitrogen atoms of the porphyrin by ionic bonds, but in the haemochromogens, by covalent bonds. It is a remarkable fact that although haemoglobin is strongly paramagnetic, oxyhaemoglobin and carbon monoxide haemoglobin are not paramagnetic at all. This means that not only do the four ionic bonds of iron in haemoglobin shift to covalent bonds on combining with oxygen, but also the paramagnetic structure of free oxygen is shifted to a non-paramagnetic form. There are five unpaired electrons per iron atom in methaemoglobin, indicating ionic bonds. Its cyan compound has one unpaired electron, indicating essentially covalent bonds. Its hydroxy compound (alkaline methaemoglobin) has three, corresponding to an intermediate type of bond.

Concerning the mechanism of the catalysts involved in oxidation, a general theory both for catalase and peroxidase is proposed by Weiss. In this the intermediate production of free radicals of the type OH , O_2H and O_2^- , and the valence change of iron play the dominant role. Another model for a peroxidase is dealt with by Krause and collaborators. A certain amorphous ferric hydroxide catalyzes the oxidation of formic acid and other aliphatic acids by hydrogen peroxide to carbon dioxide and water. The authors assume a cycle between ferric hydroxide and ferric peroxide, without change of valence of the iron. These theories, though containing useful suggestions, do not have regard for the very specific behavior of the true enzymes. These are considered in the following papers.

Stern (1) confirms the theory that catalase is an iron-porphyrin compound with a specific protein. He shows that this protein can be replaced by pyridine (and other compounds). The pyridine com-

pound is identical with that of blood haemin. He reports that catalase cannot be oxidized by oxidizing agents, and cannot be reduced by reducing agents. Since the haemin group combines with potassium cyanide, hydrogen sulfide, and sodium fluoride, but not with carbon monoxide, it must be considered as a ferric compound. Somewhat different results were obtained by Keilin & Hartree (1). They found that catalase (or its azide-, or hydroxylamino- or hydrazine compound) cannot be reduced by sodium hydrosulfite, but is reduced by hydrogen peroxide, with which it first forms a complex. It can be reoxidized by oxygen, but not by ferricyanide. Thus, as far as is known, hydrogen peroxide is the only substance able to reduce catalase, and oxygen the only substance able to reoxidize it. The ferrous nature of the compound formed in presence of hydrogen peroxide is evidenced also by the fact that compounds of catalase formed in the presence of hydrogen peroxide with sodium azide, hydroxylamine, or hydrazine, combine with carbon monoxide and are oxidized by oxygen.

Haurowitz *et al.* showed by Heyrowski's polarographic method that hydrogen peroxide is activated only by haemins, not by metal-free porphyrins. They demonstrate a hydrogen peroxide-haemin compound of green color which, though not stable in water, is stable in pyridine. It is a ferri compound. They discuss the close relationship of the catalase and peroxidase mechanisms, depending largely on the solvent and the substrate. The fundamental reaction is always the catalytic reduction of hydrogen peroxide to two molecules of water. If the reduction is performed by another molecule of hydrogen peroxide, we have catalase effect, if by another substrate molecule we have a peroxidase effect. Such a mechanism apparently does not involve the reduction of the iron of catalase, but the results presented by Keilin & Hartree are strong evidence that this reduction does occur.

In another paper Haurowitz adds that the combination of hydrogen peroxide with haemin leads not only to the catalase destruction of hydrogen peroxide but also to a peroxidase destruction of haemin. He obtained from pyridine-haemin with hydrogen peroxide in the presence of hydrogen donors green chlorophyll-like derivatives.

Stern (2), using a spectroscopic method, has described the formation of a compound of catalase with monoethylhydrogen peroxide. It gradually disappeared by a slow reaction which he believes is comparable to the fast reaction with hydrogen peroxide. Keilin & Hartree doubt that this peroxide is split by catalase at all and think that the disappearance of the compound is a secondary reaction.

Keilin & Mann showed that peroxidase from horse-radish is also a haemin compound characterized by a spectrum of the methaemoglobin type. It contains iron and can be reduced by sodium hydrosulfite. A boiled solution gives ordinary haemochromogen. The reduced form combines reversibly with carbon monoxide, the oxidized with sodium fluoride, potassium cyanide, and hydrogen sulfide. Each compound has characteristic bands. Peroxidase combines with either one or two molecules of hydrogen peroxide, according to concentration, to form compounds with characteristic spectra. Neither of these combines with carbon monoxide, and both are reduced by sodium hydrosulfite to a typical peroxidase haemin. These two hydrogen peroxide complexes decompose rapidly, liberating free peroxidase. In presence of the natural or added artificial acceptor the decomposition is even faster. A peroxidase of the same optical and chemical properties occurs in milk. The results with this group of haemin compounds show that the same haemin nucleus can combine with three different specific proteins to form either methaemoglobin, catalase, or peroxidase.

Another peroxidase-like mechanism has been found by Huszák. It involves the phenylbenzopyrane dyestuffs (flavones, flavonoles, and flavanones). They act either in combination with glucose as glucosides, or free, provided they contain two free hydroxyl groups in the phenol ring. This reaction is specific in so far as they are one hundred times more active than ordinary polyphenols. The mechanism is this: Ascorbic acid is oxidized by its specific oxidase, giving rise to hydrogen peroxide; this oxidizes the flavone which, in its turn, oxidizes another molecule of ascorbic acid, so that oxygen is entirely reduced to water by this mechanism.

As stated above, Kubowitz (1) has prepared a purified oxidase from potatoes. He finds the action of this preparation to be inhibited by carbon monoxide. This is in agreement with earlier results of Keilin. However, other oxidases have been studied and found not to be inhibited by carbon monoxide. Thus, Yakushiji has studied an oxidase from the fungus, *Lactarius piperatus*, and found it to be sensitive to potassium cyanide, but not to carbon monoxide. The preparation is quite non-specific and acts upon a number of related substrates. Yamaguchi has studied the oxidation of various phenols and phenylenediamine by *Bacillus pyocyaneus*. He has extracted the oxidase concerned and gives an interesting discussion of it. It is not inhibited by carbon monoxide.

Joyet-Lavergne shows that a dilute suspension of basic cobaltous chloride, slightly pink in color, penetrates living cells and stains the mitochondria deep green. He concludes that the mitochondria contain a catalyst which brings about the oxidation of the cobaltous ion to the cobaltic, taking it for granted that the green color indicates a cobaltic compound. He builds an extensive hypothesis about the importance of the mitochondria for respiration. A more acceptable explanation for this nice staining effect seems to the reviewers to be that mitochondria contain a substance which forms a green complex with cobalt. It may be recalled that the cobaltous complex of cysteine or glutathione is deep green, and that Joyet-Lavergne himself shows by another method that mitochondria contain glutathione.

Lyman & Barron find that glutathione is not oxidized by oxygen without a catalyst. Copper salts, haemin, or haemochromogens can serve as catalysts.

Ghosh & Rakshit claim that, although copper is acknowledged to be a catalyst for the autoxidation of ascorbic acid, a definite residue of autoxidizability is observed in the absence of copper, and this can be inhibited by sulfhydryl or disulfide compounds. The autoxidizability of certain other compounds is similarly inhibited. They resort to the hypothesis that sulfur atoms combine by co-ordinate bonds with active oxygen molecules and release them again as ordinary oxygen molecules.

Rabinowitch & Weiss describe experiments which they interpret as a reversible oxidation of chlorophyll by ferric chloride. In a methanol solution of chlorophyll the red absorption band disappears after adding ferric chloride, and reappears on addition of ferrous chloride. From this the potential range is considered to overlap with that of the ferri-ferro system. When a solution of chlorophyll and ferric chloride is kept in the dark, the band, which first disappears, gradually returns. This is interpreted by the assumption that chlorophyll acts as a catalyst in the oxidation of the solvent, methanol, by Fe^{+++} , thus giving rise to Fe^{++} .

Respiration.—An attempt to measure the metabolism of the elementary bodies of vaccinia has been reported by Parker & Smythe. No significant metabolic activity could be detected under the various conditions employed.

Korr has studied the effect of temperature on the respiration of unfertilized and fertilized sea-urchin eggs. He concludes that unfertilized eggs probably respire by means of a non-ferrous autoxidi-

zable carrier and that on fertilization the cytochrome system comes into operation. This is in agreement with some previous results.

Steir & Stannard have studied the endogenous respiration of bakers' yeast at different temperatures and have presented a theoretical discussion of their results. Emödi & Sárkány have also presented a theoretical treatment of oxygen utilization by yeast.

Meiklejohn has measured the oxygen uptake of suspensions and cultures of a free living, but unnamed, bacterium. The oxygen uptake per cell reaches a peak forty-eight hours after inoculation and drops rapidly after the number of bacteria becomes maximal. Wynd reports that the respiration of *B. coli* exhibits two phases of activity, a small but definite break in the curve always occurring. Aubel has presented a discussion of the role of nitrates in biological oxidations, particularly in *B. coli*. Barron & Jacobs have tested the effects of prontosil and prontylin on the metabolism of four different bacteria. They had a small inhibiting effect on the oxidations of *B. Friedlander* and no effect on the others. Nilsson has studied the oxidation-reduction systems in *Azotobacter chroococcum* and compared them with yeast. When cultivated on different media different enzyme systems are readily formed. Irving has published a number of his observations on the respiration of the beaver and Pomerat & Zarrow have given an interesting report on the measurement of the respiration of the newt. Davis has measured the effect of advancing age on the oxygen consumption of rats. He finds a continuous decline throughout life, the most rapid decline being during the first four months.

Lundegårdh has presented a further study of what he has called the anion respiration in plant roots. Craig has measured the respiratory quotient of seedlings of *Lupinus albus* during the early stages of germination. The results indicate that fat is not the first substrate mobilized. The effect of lactate on the respiration and photosynthesis of *Chlorella* was tested by Tang. Both functions may be reversibly inhibited but the respiration is much more sensitive.

Ponder & Macleod have studied the potential and the respiration of frog skin and the effect of carbamates and lysins upon them. The two functions do not always change in the same way.

Tyler has compared the rates of development and of oxygen consumption of tight membrane and normal echinoderm eggs. He finds the same oxygen consumption in spite of different rates of development.

Shapiro has found the oxygen consumption of the axones to be

four to five times higher than that of the sheath in resting *Limulus* optic nerve. Bracket & Shapiro have found the oxygen consumption of the dorsal lip half of intact amphibian gastrulae to be 47 per cent higher than that of the ventral half.

Cohen & Gerard have investigated the oxidizing enzymes present in brain and have compared hyperthyroid and normal brain. They conclude that the concentration of enzymes is increased in the hyperthyroid cases and that the dehydrogenases are increased relatively more than the oxidases.

Stotz, Harrer, Schultze & King have studied the respiration of normal and scorbutic guinea-pig liver and kidney. The oxygen consumption and carbon dioxide production of liver were found to be increased with the onset of scurvy, but the kidney showed no change. The anaërobic lactic acid production of each tissue and the aërobic lactic acid production of liver were found to be unchanged. Vitamin C, added to the excised tissues, was without effect. These results seem to be in contrast to those reported by Scoz *et al.*

Schorr has reported that cardiac tissue removed from a depancreatized dog and incubated at 37.5° C. in a sterile Ringer-phosphate-glucose solution for five or ten hours becomes quite like normal cardiac tissue so far as its respiratory quotient and its response to glucose is concerned.

A detailed study of the effect of pyocyanin on the metabolism of cerebral cortex was made by Young. With the addition of a substrate and in oxygen, various concentrations of dye caused an initial increase in oxygen consumption. In many cases this was followed by an irreversible inhibition. The increase was inhibited by potassium cyanide. The presence of 0.1 *M* potassium chloride did not change the effect of the dye. A concentration of 1×10^{-3} or 2×10^{-3} *M* increased aërobic glycolysis and oxygen consumption. A concentration of 2×10^{-4} or 4×10^{-4} *M* had no effect on aërobic glycolysis.

Michaelis, Moragues & Smythe have examined the effect of 21 dyes on the aërobic fermentation of yeast extract. In keeping with previous results they find that a number of them inhibit the fermentation of glucose without inhibiting the fermentation of hexosediphosphate. No correlation between the effect of the dye on fermentation and on oxygen consumption was found. There was also no satisfactory correlation between the effect of the dye and its constitution. Meyerhof, Kiessling & Schultz have suggested that the probable effect of a dye such as Rosindulin G G (which inhibits glucose fermentation

and permits the accumulation of hexosemonophosphate) is to inhibit Warburg's protein-A. The suggestion that the action of the dye is primarily directed toward a protein is very suggestive; however, inhibition of protein-A, as its function is now understood, would not explain the results. The potential range of the dye is of no direct importance though it may have an indirect effect in those cases where the leuco dye has a different affinity for the protein than the oxidized dye.

A report on the oxygen consumption of the rabbit lens and the effect of 2,4-dinitrophenol thereon was published by Field *et al.* The findings concern the oxygen consumption of lens and its measurement and show that dinitrophenol may increase or decrease this consumption depending on its concentration. In another investigation Martin & Field tested the effect of dinitrophenol on excised rabbit muscle. They found the undissociated compound to be the active agent in stimulating or inhibiting oxygen consumption. Tyler & Horowitz have studied the effect of substituted phenols on sea-urchin eggs. They believe that the effectiveness of the different added compounds in stimulating respiration and reversibly blocking cleavage is proportional to the concentration of undissociated molecules that pass through the membrane. Once through, they believe the molecules dissociate again and act in that form.

Krahl *et al.* have studied the effect of some substituted phenols at different carbon dioxide tensions. They conclude that oxidative stimulation may be favored by high intracellular concentration of the dissociated form while inhibition of oxidation and the reversible blocking of cell division may be favored by high intracellular concentrations of the undissociated form.

Hall & Culver find that the hyperglycemia which normally follows dinitrophenol administration is prevented by cutting the splanchnic nerves. Bryan *et al.* tested the effect of dinitrophenol on a depancreatized dog. They conclude that there are no striking alterations of the abnormalities of diabetes mellitus.

The possible role of phospholipids as oxygen carriers was investigated by Bloor & Snider. The phospholipids from liver or muscle had no effect on a sensitive preparation of reduced methylene blue, but if they were exposed to air for twenty-four to forty-eight hours they became water-soluble and acquired a marked oxidizing effect.

Green & Richter have presented an interesting paper on adrenaline. They show that its effect in the coupling of dehydrogenases and

increasing oxygen consumption, observed under some conditions, is due to the formation of a dyestuff from oxidized adrenaline by ring closure. They suggest the name adrenochrome for this dye. It is inactive as a vasoconstrictor, but it may play a role in the other effects of adrenaline.

Bodine & Boell find that the methylene blue-stimulated respiration in diapause and developing grasshopper embryos as well as the normal oxygen uptake of developing embryos are inhibited by carbon monoxide. In three papers Moruzzi has reported on erythrocyte respiration with methylene blue in the presence of glucose and some intermediate products of its metabolism. His conclusion seems to be that the substrate oxidized is a three-carbon molecule that in the glycolytic mechanism is a precursor of pyruvic acid.

Anderson & Alt find that, although the thyrotropic pituitary hormone has no effect on the oxygen consumption of liver or kidney tissue, it causes a distinct increase in the case of dog-thyroid tissue. Victor & Anderson report that theelin or dihydrotheelin added to rat anterior pituitary lobe *in vitro* causes an increased oxygen consumption. These compounds have no effect on liver or kidney and thyroxin has no effect on the pituitary.³

The effect of certain narcotics on tissue oxidations was studied by Jowett & Quastel (2). The effect varied with the substrate and tissue tested. Concentrations which produced narcosis *in vivo* also measurably inhibited the respiration of brain cortex. The same authors studied the effect of ether (3) and hydroxymalonate (1) on brain oxidations. The inhibitory effect of ether, at its anaesthetic concentration, on the oxidation of glucose by cerebral cortex fell within the limits of error. Hydroxymalonate had a greater inhibiting effect on the oxidation of lactate than of glucose or pyruvate. They conclude that glucose can be oxidized without first forming lactate.

Clark *et al.* have continued the study of the isolated frog heart poisoned with iodoacetic acid. They find that the exhausted heart cannot be revived by glucose or glycerophosphate but can be by lactate, pyruvate, fatty acids (propionic to decolic), alanine, and glutamic acid.

Saslow has studied the oxygen consumption and the respiratory

³ Canzanelli *et al.* report that thyroglobulin increases the oxygen consumption of liver *in vitro*, whereas thyroxin does not. They conclude that the latter is not the real hormone.

quotient of caffeinized muscle. Caffeine, in accord with earlier work, increases the oxygen consumption from four to ten times. The respiratory quotient is 1.00.

Bernheim, Bernheim & Michel have studied the action of *p*-amino-phenol on tissue oxidations. They find that it specifically inhibits xanthine oxidase.

Goldfelder has reported that an experimental sheep serum, previously found to inhibit cell proliferation *in vitro*, had no inhibitory effect on cell respiration.

U. S. Euler has studied the effects of phosphate, osmotic pressure, and pH (1) and of calcium ion (2) on the respiration of muscle preparations. She finds that at constant pH and optimum osmotic pressure there is no specific effect of phosphate. The effect of calcium on intact muscle is much greater than on muscle extract. Euler & Liljestrand have studied the effects of cyanide and conclude that in part the effects are due to acid formation. Presnell has reported findings on the respiration of skin which he believes is influenced by vitamin D.

Pistor has used a desirable modification of the usual Thunberg experiment. Instead of observing the end point of the decolorization he follows the course of the reaction in a stufophotometer. Stefanelli has described a new form of microrespirometer.

The C₄ dicarboxylic acids.—Work on the catalytic role of the C₄ dicarboxylic acids in muscle respiration has been actively pursued during the current year. The early view (cf. Banga & Szent-Györgyi) that fumaric acid was directly dehydrogenated to oxaloacetic acid is changed. Laki *et al.* and Laki (1) now believe that the fumaric is first hydrated to malic and that this is then dehydrogenated, in conformity with the view expressed earlier by Green. Das has studied malic dehydrogenase and concludes that it is identical with lactic dehydrogenase. This is contrary to the conclusion of Green, and Das attributes the discrepancy to the fact that Green did not remove the toxic oxidation product, oxaloacetic acid, completely enough and did not work at optimum concentrations. Laki (2) has studied the oxidation-reduction potential of the malic-oxaloacetic system and finds it to be -0.169 v. at pH 7.0 and 38° C. This is much more negative than the succinic-fumaric system (-0.011 v.) and approximately equal to the lactic-pyruvic system. In spite of this difference in potential, oxaloacetic acid is reduced by muscle much more rapidly than is fumaric. Under aerobic conditions it appears that the muscle has

available two hydrogen acceptors, oxaloacetic and pyruvic acid, both of which are activated by the same enzyme. Under the conditions studied by Laki (3) the oxaloacetic acid plus enzyme reacts with leuco neutral red a little more than twice as rapidly as pyruvic acid plus the same enzyme. Under anaërobic conditions it is believed that the oxaloacetic acid would not arise and thus only pyruvic would be available as acceptor. Das and Szent-Györgyi state that this difference in available acceptors under aërobic and anaërobic conditions may be involved in the Pasteur reaction. The same idea seems to be favored by Parnas & Szankowski. Although this difference may well be a pertinent factor it is not clear to the reviewers how it could be of any great importance so long as the oxaloacetic acid acts only as a hydrogen acceptor, as their theory indicates. Banga reports that the dehydrogenation of malic acid, using fumaric acid as the acceptor (a carrier is indispensable), is readily catalyzed by the yellow enzyme of Warburg. She believes this to be the function of the yellow ferment in muscle respiration. Szent-Györgyi summarizes their view as follows: Hexosediphosphate, or some product derived from it, is dehydrogenated; the hydrogen passes first to oxaloacetic acid, then to the yellow enzyme, then to fumaric acid, then to the iron-containing catalysts, and finally to oxygen. This indicates that only succinic acid (cf. Straub) reacts with the iron-containing catalysts. Krebs (2) believes, however, that at least in the case of *B. coli*, lactic, formic, and malic acids may also react directly with "activated oxygen." He also discusses the possibility of carbon dioxide serving as a hydrogen acceptor. *B. coli* may not be comparable with Szent-Györgyi's muscle preparations for Califano & Banga are of the opinion that the C_4 dicarboxylic acids do not serve as oxidation catalysts in this organism. The oxidative removal of the C_4 dicarboxylic acid from tissues has been studied by Annau & Straub. They found that if large amounts are added 20 to 30 per cent may be oxidized in eighty minutes.

Krebs & Johnson (3) have presented an interesting extension of the C_4 dicarboxylic acid catalysis by claiming that citric acid is also a catalyst for muscle respiration. This view is supported by other work (cf. Knoop; Knoop & Martius; Martius; Martius & Knoop; Orten & Smith). Martius & Knoop showed that under the influence of citric dehydrogenase citric acid was converted to α -ketoglutaric acid. They suggest three intermediate products—*cisaconitic* acid, *isocitric* acid, and *oxalosuccinic* acid. It was already known that α -ketoglutaric acid is converted by tissues to succinic acid; thus, a way for the con-

version of citric acid to a C_4 dicarboxylic acid is clear. Knoop & Martius also showed that, in a sodium carbonate solution, and on addition of hydrogen peroxide, oxaloacetic acid and pyruvic acid could condense to form citric acid and carbon dioxide. If this reaction could be made to occur biologically a way would be clear for the conversion of a C_4 dicarboxylic acid to citric acid and hence for a possible catalytic action of citric acid. The work of Orten & Smith on the precursors of citric acid in the intact animal had clearly shown that the C_4 dicarboxylic acids—succinic, fumaric, and malic—resulted in the formation of large quantities of what they considered to be citric acid. Krebs & Johnson found that under anaërobic conditions oxaloacetic acid added to muscle condensed with some unknown substance (probably of carbohydrate nature) to form citric acid. Under aërobic conditions, in the presence of arsenite, the citric acid broke down to α -ketoglutaric acid and, in the presence of malonate, to succinic acid. This offers an important change from the viewpoint of Szent-Györgyi and his school, for here oxaloacetic acid is converted by a series of oxidative reactions to succinic acid while from Szent-Györgyi's viewpoint this could be brought about only by a reduction. It seems possible that such a mechanism might well be involved in the Pasteur reaction. However, Breusch (from Szent-Györgyi's laboratory) does not accept the results of Krebs & Johnson. He believes the catalytic action of citric acid is due to its conversion to C_4 dicarboxylic acids but denies that citric acid is re-formed. He believes the condensation reported by Krebs & Johnson occurred during the neutralization of the acetoacetic acid before it was added to the muscle.

Fischer & Eysenbach have described what they call a fumaric acid hydrogenase from yeast. They believe this to be an enzyme distinct from the known succinic dehydrogenase. It catalyzes the reaction between fumaric acid and reduced dyestuffs, such as lactoflavin. As a result of this reaction succinic acid is formed, but the enzyme does not bring about an equilibrium between fumaric and succinic acids. It apparently has no effect upon succinic acid.

The oxidation of succinate by tissue preparations and the effect of various inhibitors has been studied by Potter & Elvehjem. Stotz & Hastings have studied a fumarase-free succinic dehydrogenase preparation and tested the effect of certain dyes and other agents upon it. Rosenthal finds the oxidation of succinate by liver to be much greater than previous work would indicate and recommends succinate oxidation as a method of testing for the oxidase activity of

a preparation. Libbrecht & Massart report that oxygen under pressure inhibits succinodehydrogenase.

Phosphopyridine nucleotides.—The chemistry and action mechanisms of the two pyridine codehydrogenases were adequately reviewed in Volume VI. It was reported there that cozymase acts as a hydrogen carrier in fermentation. That it acts in the same capacity in muscle has been shown by Euler, Adler, Günther & Hellström and by Meyerhof & Ohlmeyer. The question of whether it can also act as a phosphate carrier has led to confusing results (cf. Euler & Adler; Euler, Adler, Günther & Vestin; Ohlmeyer & Ochoa).

The conversion of codehydrogenase I (cozymase; diphosphopyridine nucleotide) into codehydrogenase II (Warburg's coferment; triphosphopyridine nucleotide) by an enzymatic method has been reported by Vestin. The same conversion was obtained by Schlenk by means of POCl_3 .

Euler, Adler & Eriksen find that the dehydrogenase of glutamic acid requires triphosphopyridine nucleotide for its action. They point out that in addition to this system, hexosemonophosphate (and its further oxidation products) and glucose, with their appropriate dehydrogenases, are the only systems known that work with triphosphopyridine nucleotide. All the other known systems that require coferment require the diphosphopyridine nucleotide. The glucose system works with either coferment. There are, according to Euler, Adler & Günther, two enzyme systems that act on α -glycerophosphate. One of these requires diphosphopyridine nucleotide and the other requires no coferment.

Euler & Schlenk have given the details of their preparation of pure cozymase from yeast, and Ochoa has described a preparation from muscle. In other papers from Euler's laboratory the influence of alkali upon cozymase and the enzymic destruction of diphosphopyridine nucleotide by muscle have been studied (cf. Euler, Heiwinkel & Schlenk; Schlenk *et al.*; Vestin & Euler).

Green and his coworkers [Dewan & Green (1); Green & Dewan; Green, Dewan & Leloir] have continued their studies on the coupling of dehydrogenase systems. They have shown that for a number of systems diphosphopyridine nucleotide can act as carrier. They have also produced evidence for the existence of a coenzyme oxidase, and very recently [Dewan & Green (2)] have reported that this consists of a coenzyme dehydrogenase, cytochromes *-a* and *-b*, and cytochrome oxidase. From the equilibrium of the cozymase with the β -hydroxy-

butyric acid system they estimate the normal potential of the coenzyme to be about -0.28 v. at pH 7.0. No one has succeeded as yet in titrating this system electrometrically. Karrer, Schwarzenbach & Utzinger report some measurements on two phenyldihydropyridines, but the potentials were not satisfactory. Unpublished experiments from the reviewers' laboratory on N-methylnicotinic acid amide were also unsatisfactory, but they at least indicate a very negative potential range.

Boylard *et al.* have found that cozymase and adenylic acid are rapidly destroyed by tumor extracts. They believe this to be the reason that previous tumor extracts did not glycolyze. If sufficient of these substances are added the extract can be made to glycolyze as rapidly as the tissue from which it was prepared.

A protein which acts as a catalyst for the alcohol-acetaldehyde system has been obtained in crystalline state by Negelein & Wulff. They give a number of details concerning its interaction with diphosphopyridine nucleotide and with the substrate.

Oxidation and phosphorylation.—It has been apparent for some time that in various systems a connection exists between oxidation and the synthesis of organic phosphate. This coupling has been investigated by a number of workers (Dische; Innes; Lennerstrand; Meyerhof; Needham & Pillai) during the year, but so far no one has presented a mechanism for it. Normally, the oxidation concerned with the coupling seems to be the oxidation of triosephosphate by acetaldehyde (in yeast) or pyruvic acid (in muscle) with the catalytic aid of cozymase. Needham & Pillai found that the dismutation of two molecules of triosephosphate, the oxidation of triosephosphate by oxaloacetic acid, or the oxidation of α -glycerophosphate by pyruvic acid may be coupled with phosphorylation, whereas the oxidation of glyceraldehyde by pyruvic or oxaloacetic acids is not so coupled. An interesting finding in their report is that arsenate, which does not prevent the oxido-reduction, does prevent the coupling. This may explain the effect of arsenate on fermentation.

Pasteur reaction.—Two reviews of the Pasteur reaction have been published during the year (Burk; K. C. Dixon).

Kempner reports that in a number, but not all, of isolated cells tested the respiration decreased with lowered oxygen tension, but the rate of lactic acid formation was as low as in air. He concludes that the disappearance of lactic acid formation in the presence of oxygen is not dependent upon the rate of respiration, but depends directly on

oxygen tension. Very similar results are reported by Schlayer who finds that if the oxygen consumption of goose erythroblasts is inhibited 70 per cent by lowering the oxygen tension, they still produce no lactic acid, but if the oxygen consumption is lowered 62 per cent by hydrogen cyanide the lactic acid production is 71 per cent of the anaërobic production. He concludes that respiration and glycolysis are entirely independent reactions, that oxygen inhibits glycolysis by combining with the glycolytic ferment, and that cyanide exerts its effect by preventing the combination of oxygen, not only with the respiration ferment but also with the glycolytic ferment.

Somewhat in contrast to the above results, Laser (1) has measured the respiration of retina, chorion, allantois, liver, and tumor, and except in the case of liver finds the respiration to be the same in 5 per cent oxygen as in 100 per cent oxygen. He finds, however, that aërobic glycolysis is increased by low oxygen tension. Laser (2) also reports that carbon monoxide does not affect the respiration of retina, allantois, chorion, or liver, as previously believed, but does increase the aërobic glycolysis to the anaërobic level (complete inhibition of Pasteur effect). This effect of carbon monoxide is reversed by light.

Fox *et al.* measured the oxygen uptake of several ephemerid nymphs at different concentrations of oxygen. The different species were affected very differently. In some the respiration dropped as soon as the medium was less than saturated with oxygen while in others it did not change until the concentration dropped to one-fifth saturation.

Mendel reports the interesting finding that 0.01 *M* ferricyanide inhibits the aërobic glycolysis of tumors, but has no effect on the anaërobic glycolysis. This effect is specific for tumor and does not apply to kidney.

Baker has studied the effect of glutathione on the aërobic glycolysis of tumor, brain, testes, and embryonic tissue. No significant effect could be demonstrated.

Runnström *et al.* (2) find that the addition of cysteine to bakers' yeast has a marked effect on the aërobic utilization of glucose. They report a drop in oxygen consumption and a large increase in fermentation. This is not the usual Pasteur effect for the utilization of glucose increases about three-fold. In contrast to this Machlis & Blanchard, working with three different yeasts, find that the addition of glutathione or cysteine to the medium has no effect on the metabolism. Runnström *et al.* (2) report that the presence of cysteine also

seems to increase the speed with which fluoride penetrates into the cell. In a previous paper (1) the authors reported that respiring yeast cells are much more impermeable to fluoride than are anaërobic fermenting cells. Such a result fits in well with the theory of Dixon on the Pasteur reaction. However, Hunter working with beef erythrocytes could find no change in permeability for lipoid-insoluble non-electrolytes, ammonia, or acetic acid on changing from aërobic to anaërobic conditions.

Needham, Nowinski, Dixon & Cook have studied the Pasteur reaction in embryonic tissue and conclude that the Meyerhof cycle does not apply. Imanaga, from Meyerhof's laboratory, reports that dihydroxyacetone or glyceraldehyde is condensed to hexose by liver slices under aërobic conditions but not under anaërobic conditions.

Fromageot & Chaix have discussed the respiration and fermentation of *propioni bacterium pentosaceum*. They believe that some catalyst is inactivated by oxidation under aërobic conditions. Lennerstrand (1) suggests that the Pasteur effect is due to a blocking of the acceptor function of adenylic acid, i.e., under aërobic conditions the adenylic acid is kept almost entirely in the form of its triphosphate.

Sulfhydryl groups.—A determination of the sulfhydryl oxidation-reduction potential from thermal data has been reported by Borsook, Ellis & Huffman. Their value of -0.390 volts for the normal potential at pH 7.0 is considerably lower than previous determinations.

Holtz & Triem have found that peroxide is produced in the autooxidation of sulfhydryl groups and ascorbic acid. This may be of some importance in interpreting the behavior of systems involving such groups.

A review of the reversible inactivation of certain hydrolytic enzymes has been published by Hellerman. Gemmill & Hellerman have reported the reversible inhibition of muscle glycolysis which they tentatively interpret on the basis of a sulfhydryl-disulfide.

The isolation of labile antigens of *Streptococcus hemolyticus*, their inactivation by mild oxidation, and their reactivation by reduction have been reported by Mudd *et al.*

Stern & White and White & Stern have reported on the formation of sulfhydryl groups in insulin by reduction. A preparation in which they could detect 2 to 3 sulfhydryl groups had approximately 50 per cent activity. No change was found in the other properties examined. The sulfhydryl groups could be removed by reoxidation, but the product was almost completely inactive.

Micheel *et al.* in an interesting paper on cobra poison are inclined to interpret their results on the basis of a reversible thiolactone formation, an interpretation that may have a bearing in some other cases.

The effect of oxidizing and reducing agents on phosphatases has been studied by Thannhauser *et al.* and by Pyle *et al.* Itoh & Nakamura report that the splitting action of lipase is activated by reducing agents and inhibited by oxidizing agents while the synthetic action is affected in the reverse way by these reagents.

Schöberl & Ludwig have discussed the cleavage of the disulfide bond by sulfite and potassium cyanide and the colorimetric determination of sulfhydryl and disulfide groups. They point out that the sulfhydryl formed by the cleavage of disulfide may be reoxidized to disulfide in the color-forming reaction, and in the presence of excess cleaving agent will react again. Todrick & Walker have reported a method of determining sulfhydryl groups in proteins and have applied it to several proteins.

Mawson found that dialyzed tissue extracts, heated to 50 to 55° C., will form lactic acid from hexosediphosphate if glutathione is present, but not in its absence. He believes that the precursor of lactic acid is not methylglyoxal but Needham & Lehmann suggest that it is. Chaix & Fromageot claim that sulfur must be present before certain bacteria can glycolyze.

Ketonic acids.—Krebs & Johnson (1) have published in more detail their work on the dismutation of pyruvic acid to lactic acid, acetic acid, and carbon dioxide and extended it to other keto acids. Krebs (1) has shown that the utilization of pyruvic acid by gonococci and staphylococci is due to this dismutation, the oxygen consumption observed being due to the oxidation of the lactic acid so formed. This is in contrast to the interpretation given by Barron (2) to his results with these organisms. The same dismutation of pyruvic acid has been reported by Lipmann (2) and by Elliott, Greig & Benoy. Other products of the pyruvic acid utilization reported by Krebs & Johnson (1) were succinic acid and β -hydroxybutyric acid. The formation of succinic acid from pyruvic acid in kidney was also reported by Elliott & Greig and the same transformation was found for brain tissue by Weil-Malherbe (1).

Benoy & Elliott report a synthesis of carbohydrate from pyruvate by rat tissues. The oxidation of pyruvic acid by brain was studied by McGowan and McGowan & Peters. They found it to be incompletely burned and believe that the pathway of utilization, catalyzed

by vitamin B₁, does not include succinic, α -ketoglutaric, or acetoacetic acids.

Annau & Mahr studied the dehydrogenation of pyruvic acid by preparations of muscle and kidney. The dehydrogenation required an enzyme and a coenzyme. Cedrangolo studied the oxidation of pyruvic acid with a liver enzyme. He concludes that it is first decarboxylated to acetaldehyde which is then oxidized to acetic acid. Lipmann (3) studied the dehydrogenation of pyruvate by an acetone preparation of *Bact. delbrückii*. He found it to be oxidatively decarboxylated to acetic acid and carbon dioxide. Although the prosthetic group of the dehydrogenase is cocarboxylase (vitamin B₁ pyrophosphate) the preparation cannot decarboxylate the pyruvate.

The formation and removal of pyruvic acid by embryonic tissue is discussed by Needham, Nowinski, *et al.* A metabolic disorder in which the patients excrete phenylpyruvic acid has been investigated by Penrose & Quastel. Brown *et al.* report that *Clostridium butyricum* dissimilates pyruvic acid to butyric acid, acetic acid, carbon dioxide, and hydrogen. Under certain conditions butyl alcohol is formed.

The utilization of acetopyruvic acid (diketovaleric acid) by muscle, kidney, testes, and brain was reported by Krebs & Johnson (2). Aërobically, the liver converts it to acetoacetic acid and, anaërobically, to β -hydroxybutyric acid.

In their previously cited paper Green, Dewan & Leloir reported that in the presence of tissues or tissue preparations β -hydroxybutyric acid and acetoacetic acid form a reversible system. They found the normal potential at pH 7.0 to be -0.282 v. Weil-Malherbe (2) finds that there is also an enzyme in tissues that catalyzes the oxidation of α -hydroxyglutaric acid to α -ketoglutaric acid and that these two also form a reversible system. The normal potential is, however, much more positive, i.e., -0.07 v. at pH 7.0.

An interesting interaction between α -keto acids and amino acids in which the amino group shifts to the keto acid is reported by Braunstein & Kritsman. The exchange is catalyzed by an enzyme present in a number of tissues and the only limitation seems to be that one of the acids concerned must be dicarboxylic.

Oxidation of various substrates.—Warburg & Christian have continued their study of the oxidation of hexosemonophosphate. By using their protein fractions they can cause this oxidation to occur in steps, apparently leading to a three-carbon phosphate ester as end-product. The role that this oxidation may play in fermenta-

tion and glycolysis is not clear. They describe an interesting complete oxidation of glucose or fructose coupled with the above monophosphate oxidation.

The problem has never been settled whether the so-called hexosediphosphate dehydrogenase really acts on the diphosphate molecule or whether a splitting of the molecule precedes the oxidation. Smythe has attempted to prepare an oxidized hexosediphosphate as the result of such an enzyme action but the attempt was unsuccessful and the data indicate that a splitting precedes the oxidation.

The oxidation of amines has received considerable attention. Pugh & Quastel have studied the oxidation of aliphatic amines by brain and kidney. Butyl, amyl, isoamyl, and heptyl amines are readily oxidized. The lower amines are less readily, or not at all, attacked. Mann & Quastel have studied the oxidation of choline by rat liver. It is oxidized to betaine aldehyde and possibly to betaine. Blaschko *et al.* have studied the enzymic oxidation of amines and conclude that the enzyme concerned is the same as adrenaline oxidase. Richter finds that this adrenaline oxidase forms the corresponding aldehyde and methylamine from adrenaline. Philpot and Kohn have studied the oxidation of tyramine. The products formed are the corresponding aldehyde, ammonia, and hydrogen peroxide. The enzyme required is an aerobic oxidase that requires no coenzyme. Kohn also concludes that this enzyme is identical with adrenaline oxidase.

Kuhn, Köhler & Köhler have fed highly unsaturated fatty acids to rabbits and have isolated from the urine the corresponding dicarboxylic acid. The yield is greatly increased if the amide, the methylamide, or the anilide is fed. Franke & Jerchel and Hinsberg & Ammon have studied the autoxidation of unsaturated fatty acids. Califano has studied the oxidation of fatty acids in fatty livers. He believes that the fat dehydrogenases are specifically inhibited in such livers. Artom has also studied the oxidation of fatty acids. He believes the ω -oxidation is followed by β -oxidation proceeding from both ends.

Franke & Hasse have studied the oxidation of oxalic acid by an enzyme preparation from *Hylocomium umbratum*. Carbon dioxide and hydrogen peroxide are formed. Franke & Lorenz have presented a detailed study of glucose oxidation by enzyme preparations. They classify the enzyme as an aerobic dehydrase.

The oxidation of mannite by yeast preparations has been studied by Müller. The oxidation of carbohydrate to glucosone by enzymatic action was studied by Bond *et al.* The oxidation of the cyclohexane

ring in the organism was studied by Bernhard. It apparently can be converted to the benzene ring if a carboxyl group is present on the ring.

The oxidative fermentative formation of melanin from tyrosine has been studied by Fürth & Thallmayer. Arnow finds that tyrosine may be converted to dihydroxyphenylalanine by irradiation with ultraviolet light. He suggests that in the animal body irradiation may play a role in the formation of this substance, which then goes on to melanin.

The dehydrogenases of the human placenta have been investigated by Thunberg. Ehrismann and Ehrismann & Dramburg have tested the oxidizability of a very large number of substrates by micrococci and streptococci. Tang & Lin (2) have tested the oxidizability and fermentability of a large number of compounds by *Saccharomyces wanching*. Johnson has tested the effect of a number of glycosides on hexose oxidation by luminous bacteria.

Leloir & Dixon have tested the effect of cyanide and of pyrophosphate on the oxidation of seven substrates by their dehydrogenases. Only xanthine oxidation was inhibited by cyanide and only succinic acid oxidation was inhibited by pyrophosphate. Dixon has also tested the effect of iodoacetate on a number of dehydrogenases. Only alcohol dehydrogenase was sensitive to the low concentrations of iodoacetate that inhibit fermentation. Adler & Sreenivasaya have found that formic dehydrogenase is also inhibited by cyanide. In this connection the remarks by Kubowitz (2) are pertinent. He has described an interesting system consisting of alcohol, pyridine-protein, catechol, copper protein, and oxygen. The pyridine-protein dehydrogenates the alcohol, the copper protein dehydrogenates the catechol, the orthoquinone which is formed reoxidizes the pyridine, and oxygen reoxidizes the copper. The last step is sensitive to cyanide and carbon monoxide so in this case an alcohol dehydrogenase is cyanide sensitive. If, however, one replaces the copper protein and catechol with alloxazine-protein (yellow enzyme) the alcohol will be dehydrogenated as before but in this case it is not sensitive to cyanide or carbon monoxide.

Reindel & Schuler find that uric acid can be reduced by tissues to xanthine. Mamoli & Vercellone have been able to reduce both carbonyl groups of dehydroandrosterone by the use of fermenting yeast. Langlykke *et al.* report that *Clostridium butylicum* reduces acetone to isopropyl alcohol.

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CHEMISTRY OF THE CRYSTALLINE ENZYMES*

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Three new enzymes, ficin proteinase (20), catalase (36), and papain (19) were isolated and crystallized this year. The protein of acetaldehyde reductase has also been crystallized (29) so that the protein as well as the prosthetic group of this enzyme has now been isolated. The coenzyme of carboxylase has been crystallized and its structure determined (35). Ten¹ enzymes have therefore been obtained in crystalline form to date and, in three cases, the inactive precursors of the enzymes have also been crystallized.

A considerable amount of evidence has been presented concerning the purity of some of these crystalline preparations and it has been found, as might be expected, that they may contain more than one protein. Crystalline pepsin preparations, for instance, normally contain about 10 per cent of non-protein nitrogen and may contain up to 30 to 40 per cent of inert protein. A number of tests for the purity of the enzymes have been suggested and carried out, such as rate of inactivation compared to formation of denatured protein, sedimentation-rate determinations, diffusion measurements, electro-cataphoresis, immune reactions, inactivation by ultraviolet light, and solubility determinations. Experience in this laboratory has shown that this last method is the only one upon which reliance can be placed since several crystalline preparations have been obtained which gave constant activity and which were homogeneous by other tests but which showed the presence of more than one protein when tested by the solubility method. No case has been found, however, in which crystalline enzyme proteins contained impurities of non-protein nature, except degradation products. Thus, although convincing evidence for the purity of some of the crystalline enzymes has not yet been

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¹ Urease (Sumner), catalase (Sumner & Dounce), pepsin (Northrop), pepsinogen (Herriott & Northrop), trypsin (Kunitz & Northrop), trypsinogen (Kunitz & Northrop), chymotrypsin (Kunitz & Northrop), chymotrypsinogen (Kunitz & Northrop), carboxypeptidase (Anson), amylase (Caldwell, Booker & Sherman), yellow respiratory ferment (Warburg & Theorell), ficin (Walti), and papain (Balls, Lineweaver & Thompson).

obtained, it appears unlikely that they will be found to contain impurities other than proteins or protein split-products.

It is perhaps too soon to conclude that all other enzymes are proteins but it is evident that particular attention should be paid to the protein fraction in all enzyme-isolation work and also that the handling of relatively large quantities of material by the usual technique of protein chemistry is the most promising method of crystallization.

The oxidative enzymes, i.e., the yellow respiratory ferment, catalase, and acetaldehyde reductase, are conjugated proteins and contain a prosthetic group. These enzymes are called by Warburg "dissociating proteids." The other, purely hydrolytic, enzymes may also contain a prosthetic group but up to the present no evidence for such a group has been obtained. It seems quite certain that if such a group is present it has no characteristic absorption spectrum nor is it active when separated from the parent molecule. Identification of the hypothetical prosthetic group is thereby rendered extremely difficult.

Crystallization may change the properties of the preparation in a very striking way. Thus, amorphous chymotrypsinogen preparations, although indistinguishable by analysis from the crystalline material, are activated by enterokinase while crystalline chymotrypsinogen cannot be activated at all by kinase but is rapidly activated by small amounts of trypsin. This peculiar behavior is due simply to the fact that amorphous preparations contain minute amounts of trypsinogen. The trypsinogen is activated to trypsin by kinase and the trypsin then activates the chymotrypsinogen. Crystallization removes the trypsinogen and hence crystalline preparations cannot be activated by kinase. A similar striking change in the activation reaction takes place on the crystallization of trypsinogen.

These examples are discussed since the question of the activation of trypsinogen has been one of the most controversial and apparently complicated subjects to be found in enzyme literature for the last thirty-five years. It is apparent, therefore, that results with non-crystallized enzymes may be complicated and cannot be considered as final although they are, of course, of importance and are necessary steps in the isolation of the enzyme itself. For this reason the present review is restricted to those results which have been obtained with pure material and which, presumably, are therefore final and reproducible.

CRYSTALLINE ENZYMES
HYDROLYTIC ENZYMES

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PEPSIN

Several papers have appeared concerning the properties of crystalline pepsin. Herriott (1) has shown that the enzyme is inactivated by iodine and that di-iodotyrosine may be isolated from the hydrolyzed product, thus confirming the work with acetyl pepsin which indicated that changes in the tyrosine resulted in loss of activity. At pH 5.0 to 6.0 the tyrosine of pepsin is iodinated with a loss in enzymatic activity. When the activity is nearly zero the quantity of iodine combined is 13.4 per cent of the protein and corresponds to that amount which would combine with all of the tyrosine. After acid hydrolysis of a completely iodinated pepsin preparation, 82 per cent of the total iodine was identified as di-iodotyrosine although only 53 per cent of the di-iodotyrosine was isolated in crystalline form.

Steinhardt (2) has carefully investigated the alkali inactivation of pepsin and found that the reaction is strictly monomolecular and that at constant ionic strength the velocity is inversely proportional to the fifth power of the hydrogen-ion concentration. Previous work by Michaelis & Rothstein (3) and Goulding, Wasteneys & Borsook (4) has found reaction rates proportional to the third or fourth powers of the hydrogen-ion concentration. Goulding, Wasteneys & Borsook, using crude pepsin preparations, found that the reaction proceeded very rapidly for part of the way and then became monomolecular. It is probable that these varying results were due to complicating factors involved in the use of crude preparations.

Steinhardt has also found that when the effect of temperature on the hydrogen-ion concentration of the solution is taken into account the critical increment becomes 18,300 calories instead of 63,000. The corrected figure is in reasonable relation to the theoretical number of molecular collisions of pepsin ions, whereas the larger figure is of an entirely different order of magnitude. This work, therefore, offers a reasonable explanation for the very high figure often associated with protein denaturation. Steinhardt suggests that the rate depends on the concentration of a single pepsin ion in which five of the primary amino groups of cystine have lost their positive charges. The acidic dissociation constant of these groups is 1.7×10^{-7} . The evidence for the existence of a pentavalent ion seems quite convincing but the identification of the groups as the amino groups of cystine is not so certain. It was found, for instance, by Herriott & Northrop

(5) that acetyl pepsin which contained no free amino group was inactivated in about the same way as pepsin. Chicken pepsin is quite stable in the range of pH where swine pepsin is inactivated. On the other hand, the heat of reaction and the value of the dissociation constant do not agree with any other known groups in proteins.

Inactivation of pepsin by ultrasonic waves.—The inactivation of pepsin by ultrasonic waves was reported by Chambers (6). Solutions of crystalline pepsin are inactivated in solutions containing air but not in solutions containing nitrogen or hydrogen, or which are gas-free. The rate of the reaction is again monomolecular. Certain crude preparations show increase in activity for the first few minutes of the experiment and it is suggested that this is due either to the breaking up of colloidal particles or to the destruction of an inhibiting substance present in the crude material.

Electro-cataphoresis of pepsin solutions.—Ågren & Hammarsten (7) have found that certain crystalline pepsin preparations may be separated into an active and inactive portion by electro-cataphoresis at pH 3.3, the inactive portion going to the cathode and the active portion to the anode. It has been frequently noted (2, 8) that crystalline pepsin preparations prepared from commercial pepsin may contain considerable amounts of inert protein which can be removed only by oft-repeated crystallization, or autolysis at pH 2.0, or prolonged washing. It is probable that Ågren & Hammarsten's preparation contained a similar inert protein which is removable by cataphoresis.

Immunological relationships of pepsin and pepsinogen.—Immunological relationships of pepsin and pepsinogen have been studied by Seastone & Herriott (9). They found that anti-pepsin serum, prepared with denatured swine pepsin, reacts with pepsin from cattle or guinea pig but not with rabbit, chicken, or shark pepsin. Anti-pepsin serum reacts both with pepsin and pepsinogen but does not react with the serum proteins from the homologous species. Pepsinogen anti-serum reacts with pepsinogen but not with pepsin nor with serum proteins from the homologous species. Conversely, anti-sera made with serum proteins do not react with pepsin or pepsinogen. Evidently then, pepsin possesses two specificities—the specificity as an enzyme, since there is a cross reaction between pepsins of different closely related species but not with serum proteins of its own species, and also a slight species specificity since pepsins from distantly related species do not give cross reactions. The species specificity may be

more clear cut than the experiments show, owing to the necessity of using denatured pepsin, since it is known that denaturation decreases the specificity of protein reactions. Since pepsin does not give cross reactions with the normal serum proteins it may, in a sense, be considered a foreign protein as is the lens protein.

Brücke's "protein-free" pepsin.—Kraut & Eusebio (10) have reinvestigated the properties of Brücke's "protein-free" pepsin which has frequently been cited as an example of a protein-free enzyme. They compared the properties of pepsin prepared by Brücke's method with crystalline pepsin prepared according to Northrop. Both preparations were made from commercial pepsin. Only about 1 per cent of the total original peptic activity was recovered by Brücke's method and this fraction was quite different from crystalline pepsin. It hydrolyzed casein which had previously been digested by crystalline pepsin and hence possessed distinct enzymatic specificity. It contained less nitrogen than crystalline pepsin and gave fainter tests for protein. Brücke's pepsin, therefore, is evidently quite distinct from crystalline pepsin and represents a different enzyme of unknown chemical composition. Commercial pepsin preparations are prepared by autolysis of gastric mucosa and hence contain the cell enzymes as well as the enzymes of gastric juice; it is not surprising, therefore, that more than one proteolytic enzyme is present. An enzyme especially active on gelatin had been investigated previously by Northrop and it is quite probable that a number of other proteolytic enzymes are likewise present. Crystalline pepsin may be prepared from gastric juice and practically all of the proteolytic activity of commercial pepsin may be obtained in the crystalline product.

Structure of pepsin.—Wrinch (11) has shown that the X-ray data, molecular weight, and known chemical composition of pepsin agree with the assumption of a closed cyclol structure having as a unit 288 amino acid residues.

TRYPSIN AND CHYMOTRYPSIN

The study of proteolytic enzymes has always been complicated by the fact that the changes in structure of the substrate were unknown as well as the nature of the enzyme. The recent work of Bergmann and his collaborators on the synthesis of substances of known structure which are attacked by proteolytic enzymes has, therefore, been a great advance. Bergmann & Fruton (12) have described the synthesis of peptides which are hydrolyzed by chymo-

trypsin. It was found that this enzyme can split peptides in which tyrosine or phenylalanine supply the peptide carbonyl group. Thus, carbobenzoxyglycyl-*L*-tyrosylglycine amide is rapidly hydrolyzed by the enzyme.

Tazawa (13) has prepared a crystalline trypsin which appears from the photographs of the crystals and general properties to be chymotrypsin. It is stated that this preparation is able to hydrolyze glycyl-*D*-glutaminic acid anhydride.

Eagle & Harris (14) have investigated the coagulation of blood by crystalline trypsin. It was found that trypsin can replace platelets in the blood-clotting system and it is suggested that the enzyme changes prothrombin to thrombin. This mechanism would be analogous to the transformation of chymotrypsinogen to chymotrypsin by trypsin. Tyson & West (15) found that the clotting time of hemophilic blood was reduced by crystalline trypsin.

Schmidt (16) has obtained active trypsin from blood plasma by precipitation with trichloroacetic acid. The enzyme in the plasma appears to be combined with an inhibitor in a way similar to that found by Kunitz & Northrop for the inhibitor-trypsin compound in pancreatic extracts.

CARBOXYPEPTIDASE

Carboxypeptidase hydrolyzes chloroacetyl tyrosine, and certain peptides which contain a free carboxyl group, in such a way as to liberate the amino acid the carboxyl group of which is free in the peptide. The enzyme is active in the presence of formaldehyde which abolishes the free amino groups of both enzyme and substrate. The purification and crystallization of carboxypeptidase from autolyzed beef pancreas is described by Anson (17). The enzyme is most easily prepared from exudate of frozen pancreas. The procedure consists, essentially, in precipitation of the active globulin fraction by dilution of a slightly acid solution and extraction of this precipitate with barium-hydroxide solution. The enzyme is crystallized by careful addition of acid to the barium-hydroxide extract. The once crystallized material contains more or less tryptic activity which may be removed by repeated recrystallization. The carboxypeptidase activity remains constant through repeated recrystallization and the recrystallized material is free of dipeptidase, trypsin, and aminopolypeptidase. The loss in activity is proportional to the formation of denatured protein when the solution is heated or denatured by acid.

It was shown that the enzyme exists in fresh pancreas in an inactive form and is transformed slowly to the active form if the extract is allowed to stand in neutral solution and at 37° C. The inactive precursor was partially purified and it was found that it could be activated by trypsin in the same way as chymotrypsinogen. The crude preparations may also be activated by enterokinase but it is possible that this activation takes place through the intermediate activation of trypsinogen which is present in the crude material. Methods for determining carboxypeptidase and procarboxypeptidase are described.

Ågren & Hammarsten (18) have found that carboxypeptidase crystals which contain some trypsin may be separated from the trypsin by electro-cataphoresis.

PAPAIN

Balls, Lineweaver & Thompson (19) have reported the isolation of crystalline papain as small needles from undried latex of green papaya fruit. No change in the ratios of the activities on the substrates milk, casein, and hippurylamide was detected during five recrystallizations. A preparation which had been 94 to 97 per cent inactivated by hydrogen peroxide could be crystallized in the same crystalline form and recrystallization could be carried out without any apparent change. The inactivation could be completely reversed by the use of an activator.

FICIN PROTEINASE

Walti (20) has reported the crystallization of a catheptic or papain type protease from *Ficus* latex and has named this enzyme "ficin." The sulfur, which is 1.6 per cent of the protein, is present as sulfhydryl groups. It is inactivated by hydrogen peroxide and similar oxidizing agents and reactivated by cysteine.

REDOX ENZYMES

DISSOCIATING PROTEIDS

Knowledge of the enzymes concerned in oxidation and reduction reactions in the cell is largely due to the work of Otto Warburg and his collaborators. An excellent detailed review of this work has been prepared by Professor Warburg (21) and only the most recent papers are discussed in the present summary. The enzymes consist of a

protein (*eiweis*s) with which a prosthetic group is combined. The complex only is active and is called a "dissociating proteid." The specificity depends on the protein and different cells contain different proteins which may combine with the same prosthetic group to form enzymes of different specificity. The prosthetic group in turn contains groups which take part in the reaction and these are called "active groups." The protein part of the enzyme also combines reversibly with the specific "partner" with which the prosthetic group of the enzyme reacts in the living cell. There are thus two equilibria and two dissociation constants.

$$d = \frac{[\text{partner}] [\text{proteid}]}{[\text{proteid-partner}]}$$

and

$$D = \frac{[\text{prosthetic group}] [\text{protein}]}{[\text{protein-prosthetic group}]}$$

Protein (*eiweis*s) is used in the sense of "simple protein," i.e., one consisting wholly of amino acids.

Since only a special protein can unite with the prosthetic group to form the enzyme the protein part itself must have a special configuration which is not yet known. It appears at present that this protein part of the enzyme may be analogous to the hydrolytic enzymes themselves and that the "prosthetic groups" (coenzymes) are special devices necessary for oxidation reactions but not required for hydrolytic reactions.

PROTEIN OF THE YELLOW RESPIRATORY FERMENT

The yellow respiratory ferment was the first enzyme of this type to be isolated and crystallized (Warburg and Theorell). The enzyme is the oxygen carrier in cell respiration and also acts as the oxidative enzyme in the absence of molecular oxygen (22). Earlier work has shown that the enzyme consists of a protein combined with lacto-flavinphosphoric acid. A detailed study of this protein has been made by Theorell (23). The results indicate that the protein combines with the phosphoric acid and also with the imino group of the flavin (24). The enzyme is separated into its components by dialysis in acid solution. Under these conditions the protein is denatured and the liberated flavin dialyzes out. When this denatured protein is dialyzed against water it reverts to the native state, as shown by solubility and

by the disappearance of free sulfhydryl groups. This native protein may then be recombined with the flavin to form the active enzyme. The protein is an albumin and does not precipitate at its isoelectric point, about pH 5.78. The electro-cataphoretic pH curves of the enzyme and of the free protein have been determined. The curves are the same from pH 7.0 to 9.0 but differ in the acid range from pH 5.0 to 7.0. The difference in the curves agrees with the assumption that combination takes place between the protein and the phosphoric acid hydroxyl and one imino group of the flavin. Sedimentation-rate determinations on the protein give a constant agreeing with that previously found for the active enzyme. The protein may also be inactivated at 38° C. but the protein obtained in this way differs from that obtained by acid dialysis. It contains no free sulfhydryl groups and is soluble in neutral salt solution. The active enzyme, however, may be obtained from the heat-inactivated material so that the reaction is reversible. Theorell suggests that this property of rapid and easy reversal of denaturation is characteristic of enzyme proteins. It is true that trypsin, chymotrypsin, and pepsinogen are much more easily converted from the denatured to the native form than are most proteins. On the other hand, pepsin is quite difficult to recover in the native form while serum albumin is almost as easily transformed to the native state as are most of the enzyme proteins.

Haas (25) has found that the yellow respiratory ferment oxidizes triphosphopyridine nucleotide (cozymase, the prosthetic group of acetaldehyde reductase) faster than diphosphopyridine nucleotide. No reaction takes place if only the prosthetic group alone of the yellow respiratory ferment is added.

If excess triphosphopyridine nucleotide is added to the yellow respiratory ferment in the presence of hydrogen and hydrosulfite a red color develops. This color is due to the formation of a flavin-protein-nucleotide compound.

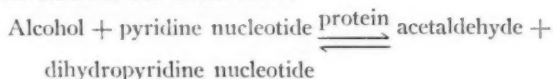
The yellow respiratory ferment also oxidizes malic acid (26). The reduced form of the enzyme may be oxidized by the fumaric acid succinoxidase system (27).

ACETALDEHYDE REDUCTASE

This enzyme catalyzes the reduction of acetaldehyde to alcohol. It is similar in general structure to the yellow respiratory ferment and consists of a protein combined with a prosthetic group, diphosphopyridine nucleotide. This prosthetic group is the cozymase of

Harden & Young and has been previously isolated and its structure determined by Warburg & Christian (28) and also by Euler. The protein part of the enzyme was crystallized this year by Negelein & Wulff (29) and the equilibrium between the protein and the prosthetic group studied. The prosthetic group exists in two forms, one of which contains pyridine and the other one dihydropyridine. The hydrogenated form combines about three times as completely with the protein as the non-hydrogenated form. The nucleotide differs, therefore, in this respect from the triphosphopyridine nucleotide which combines to the same extent in the hydrogenated and non-hydrogenated condition. The protein was obtained from Lebedew's extract of bottom yeast. Much of the inert protein was removed by heating to 55° C. and the protein then fractionated by acetone alcohol and ammonium sulfate and crystallized by means of ammonium sulfate. The protein is stable from pH 5.0 to 8.5. It is easily inactivated by heavy metals.

The effect of the concentration of protein, diphosphopyridine nucleotide, alcohol, and acetaldehyde on the velocity of the following reversible reaction was determined:



The reaction was followed by the change in light absorption of the pyridine nucleotide.

In the presence of excess alcohol the reaction rate is expressed by the equation

$$-\frac{dc}{dt} = \frac{Bc}{A - c}$$

where c = concentration of unhydrogenated pyridine nucleotide and A and B are constants independent of the time of reaction. A has the dimension: concentration of pyridine nucleotide, and B the dimension: concentration of pyridine nucleotide per minute. With the concentration of total nucleotide constant and that of the protein varying, A is independent of the protein concentration and B is proportional to the protein concentration. With the concentration of total nucleotide varying, B is constant; A increases with increase in nucleotide concentration.

The hydrogenation-dehydrogenation reaction was also studied in detail and found to agree with theory.

The equilibrium constant, Q , for the reaction is defined by the equation

$$Q = \frac{c}{c^1} \cdot \frac{A}{A^1}$$

where c = concentration of unhydrogenated nucleotide in mols per liter; c^1 = concentration of hydrogenated nucleotide in mols per liter; A = concentration of alcohol in mols per liter; A^1 = concentration of acetaldehyde in mols per liter. At 20° C. and pH 7.9, $Q = 1.35 \times 10^3$.

The chemical nature of the prosthetic group of acetaldehyde reductase, the chemical changes involved in the reaction, and the kinetics of the reaction are thus known in detail. There remains to be determined only the structure of the protein itself. The reaction is, therefore, the best understood of any enzymatic system.

COZYMASE

Diphosphopyridine nucleotide, the coenzyme of the acetaldehyde reductase just discussed, was shown by Warburg & Christian (28) and by Euler and associates (30) to be identical with cozymase.

Cozymase is known to be involved in a number of reactions which occur in muscle and in fermentation. The protein components necessary for these reactions have not yet been isolated so that the results are not so definite as in the case of acetaldehyde reductase and cannot be briefly reviewed at present. A series of interesting and important papers, largely by Euler and Meyerhof and their coworkers, has appeared in the current volumes of the *Biochemische Zeitschrift* and the *Zeitschrift für physiologische Chemie* (33). It appears that cozymase is involved in lactic acid formation (31) and also in glycolysis (32).

After treatment with alkali cozymase is said to act as cophosphorylase [Vestin & Euler (33)] but its exact relation to the transfer of phosphate groups is not yet clear.

COCARBOXYLASE

Cocarboxylase has been crystallized and its structure determined by Lohmann (34, 35). It is the diphosphoric acid ester of aneurin (vitamin B₁). One phosphoric acid group may be split off by acid hydrolysis to yield the crystalline hydrochloride. Both phosphoric acid groups may be split off by an enzymatic reaction to yield aneu-

rin. Only the diphosphoric acid ester is active as cocarboxylase. 1.7 μ g. cocarboxylase hydrochloride is a curative dose by the pigeon test. The effects of substrate, cocarboxylase, magnesium salts, hydrogen ion, and acetaldehyde concentration on the kinetics of the reaction were determined.

The fermentation of sugar by yeast extracted with alkali requires the addition of hexosediphosphate, adenylypyrophosphate, cozymase, cocarboxylase, and a magnesium salt.

The enzyme carboxylase consists of a compound of the cocarboxylase with a special protein. This special protein is as yet unknown.

CATALASE

Sumner & Dounce (36) have isolated beef-liver catalase in needle or platelet crystals. Their method of preparation consists of extraction and fractional precipitation with diluted dioxane (1,4-di-ethylene oxide) followed by crystallization of the precipitate from a chilled ammonium sulfate solution. Eight recrystallizations did not change the "*Kat f*" of 26,000. Analyses show the crystalline enzyme to be a typical protein with an isoelectric point at pH 5.7, iron content of 0.1 per cent, ash content of 0.5 per cent and absorption bands at 627 and 536 $m\mu$. The prosthetic group was crystallized after separation by the acid-acetone treatment. Its exact chemical nature is not clear but it is probably hemin or a closely related porphyrin. Catalase from horse and swine liver could not be crystallized.

METHODS

New and elegant micromethods have been described by Linderstrøm-Lang—a dilatometric method for peptidase (37) and an ingenious method for choline esterase based on the principles of the Cartesian diver (38). The interesting series of papers from Linderstrøm-Lang's laboratory dealing with the distribution of enzymes in tissues has been continued (39, 40).

BOOKS

An excellent general review of recent enzyme work has been published by Bersin (41).

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² An advance copy of this paper has been received from Professor Warburg to whom the writers are also indebted for suggestions and criticisms in connection with this review.

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THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
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THE CHEMISTRY OF THE CARBOHYDRATES AND THE GLYCOSIDES*

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CELLULOSE

It is a fact of outstanding interest that cellulose and starch, materials which are so different in appearance and behaviour, yield nothing but glucose on hydrolysis. Moreover, the glucose has the glucopyranose structure and the molecules are joined together through oxygen uniting C_1 in one unit to C_4 in another. The only difference is that the glucoside linkage is α in starch and β in cellulose, yet this suffices to make the materials so different. There can be no more striking example of the implications of stereochemical differences.

Cellulose, if for no other reason than its abundance, is of unique interest; as Sir William Bragg has said, "It is pre-eminently the molecule of growth in the vegetable world." The difficulty immediately arises: What is pure cellulose? How far is it altered during the processes antecedent to its getting into the vessels of the chemical laboratory free from other cell-wall constituents with which it is in the most intimate association?

Constitution.—It will be helpful to enumerate the reactions on which the constitution of cellulose is based.

Glucose is produced from cellulose in almost quantitative yield on hydrolysis (1). When hydrolysis is incomplete, the large molecule is not completely disrupted, and crystalline fragments are produced containing two, three, and four glucose units as well as dextrans of larger molecular size (2, 3). Exhaustive methylation produces considerable amounts of trimethylcellulose which, when hydrolysed under controlled conditions, gives rise quantitatively to 2,3,6-trimethylglucose.

It is possible to obtain a triacetate in which the fibrous structure is still maintained. On acetolysis it gives the octacetate of cellobiose in a yield which, allowing for losses, is what might be expected if cellulose were made up of a repeating unit of this size. The maximum yield of cellobiose obtained approaches 67 per cent, while under other conditions 17 per cent of cellotriose has been obtained (4).

* Received January 11, 1938.

As the series is ascended from glucose, the properties change progressively in the direction of cellulose. The identity of the linkages between the glucose units in cellulose and its oligosaccharides is indicated by the fact that the molecular optical rotation (5) is additive, being the sum of those of the two terminal units and those of the non-terminal units. Further, the kinetics of the hydrolysis of these chains, studied by Kuhn (6, 7), supports the hypothesis that cellulose consists of long chains linked throughout by the cellobiose linkage. These and other observations have been taken to indicate that cellulose may be a chain of considerable length of β -glucose units linked together by a special spatial arrangement so that the terminal carbon atom (C_6) lies alternately first on one side of the chain and then on the other, giving in effect a repeating unit of the size of cellobiose. It may be mentioned that in no instance does degradation by biological agents give rise to the formation of cellobiose from cellulose in the way that maltose is formed from starch by diastase; moreover, cellobiose is not one of the sugars found in plants.

The cellulose fibre is essentially crystalline in the sense that it has a structure possessing regularity of arrangement and orderly repetition. The fibre contains many crystals, all of which are oriented in one general direction in common along the axis of the fibre. The cellulose molecular chains lie side by side in thin bundles; they represent the attempts at crystallisation of immensely long thin molecules. The X-ray photographs show a periodicity of $10 \cdot 3 \text{ \AA}$ in the direction of orientation, which is identical with the length of two anhydro-glucose units linked together as cellobiose.

The carbon skeleton of cellulose may be written as in formula I. One terminal group in the cellulose chain is potentially aldehydic and therefore reducing. Such reducing properties are exhibited by the celloextrins obtained by partial hydrolysis of cotton cellulose (8). Cellulose itself shows no reducing properties and since most preparations have faintly acidic properties, it is generally presumed that the terminal group is oxidised to a carboxyl group. It is a question whether this happens during purification or during the exposure of the cotton hair to sunlight and oxygen on the plantation. If cotton is purified by hot alkaline treatments it naturally shows no reducing properties since such would be destroyed by the treatment, as happens with hydrocelluloses. If cotton is purified by solvent actions and mild acid treatment it does have reducing properties but here it is impossible to say that it has had sufficient treatment to be only cellulose. It

is further doubtful whether the ideal pure cellulose with a chain length of many thousand glucose units could be experimentally demonstrated to have reducing properties.

When cellulose is more strongly oxidised, the carbinol groups in the side chain are converted into carboxyl, forming acid groups of the uronic type. Such changes must happen in plants to form the polyuronic acids.

The structural significance of a terminal hydroxyl is that it may esterify with neighbouring micelles, a fact required to account for the resistance to methylation. Equally, the terminal aldehyde groups may combine by forming glucoside links with other micelles to form giant rings.

During the period 1920 to 1926, cellulose was widely held to consist of small structural units held together by some form of residual valency. Polanyi (9) had calculated that the unit cell contained four glucose residues and various theories were advanced. Clarity was first introduced when Sponsler & Dore (10) showed that the X-ray diagrams fitted in with the hypothesis that the fibres consisted of long parallel chains of glucose units linked by primary valencies.

Molecular size.—Whilst there is general agreement as to the make-up of the cellulose chain, its length is still the subject of acute controversy. Physical methods have their limitations when applied to high polymers. Undoubtedly useful application has been made of osmotic pressure, sedimentation, equilibrium, and viscosity measurements, all of which point to high molecular weights for native cellulose.

Chemical methods are based on the expectation that one or other of the end groups in a long chain molecule exhibits a specific property by which it can be estimated. Haworth (11) pointed out that one end group should be unique in yielding tetramethylglucose on methylation and determined the amount of this by a process involving acetylation, and simultaneous deacetylation and methylation to trimethyl cellulose. This was hydrolysed and the tetramethyl body separated by fractional distillation of the glucosides. A minimum chain length of 100 to 200 glucose units was found by this method, but it is evident that even very slight hydrolysis at any stage of the acetylation or methylation would considerably affect the result if the initial molecular weight were high.

Recent work seems to confirm this view. Hess & Neumann (12) found that in the presence of air a cotton cellulose, extracted first

with alcohol and benzol, then three times with 5 per cent sodium hydroxide at 20° and five times with 2 per cent sodium hydroxide at 95°, gave by the methylation method an apparent chain length of 1075 glucose units. This was increased to 2810, when twice as much dimethylsulphate was used for methylation and decreased to 285 again when eight washings with 2 per cent sodium hydroxide and five washings with 9 per cent sodium hydroxide, each at 98°, were introduced with the object of washing away such short-chain soluble products as might be present. There is clear evidence here of a degradation of the cellulose caused by alkali in presence of air.

When, on the other hand, air was carefully excluded both during extraction and methylation, seven washings with 2 per cent sodium hydroxide at 98° were judged sufficient after the alcohol-benzol treatment. Under these conditions no tetramethylglucose at all could be obtained, an indication that the length of the chain was very great. That the elimination of air prevents the degradation caused by alkali is indicated also by experiments at the Shirley Institute.

The complete methylation of cellulose to trimethyl cellulose, hitherto a matter of difficulty, since by means of dimethyl sulphate a methoxyl content of 43-44 per cent is all that can be obtained, can be achieved, according to Freudenberg & Boppel (13), by suspending cellulose in liquid ammonia and treating with sodium and methyl iodide. The product is snow-white and still possesses a threadlike structure. It has not yet had the end-group method of analysis applied to it.

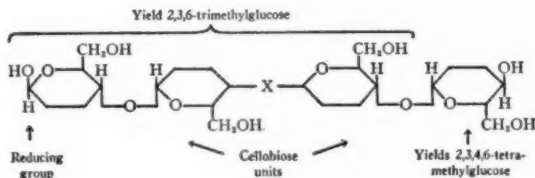
Staudinger (14), who very definitely claims that of the macromolecules of starch, glycogen, and cellulose each contains more than 1,000 glucose residues united through chemical valencies (*Hauptvalenzen*), gives figures (Table I) to show that the degree of polymerisation is not altered by conversion into triacetate and hydrolysis of this back to carbohydrate.

TABLE I
DEGREE OF POLYMERISATION OF CELLULOSE, CELLULOSE ACETATE,
AND REGENERATED CELLULOSE (STAUDINGER)

(Number of glucose residues)		
Original Cellulose	Acetate	After Hydrolysis
500	505	490
1,000	970	955
1,600	1,650	1,630

The polymerisation was determined both by viscosimetric and osmotic measurements. The figures afford strong proof of the existence of large molecules of glucose residues united by simple valencies and are opposed to the idea of physical aggregates or micelles.

Staudinger regards the colloid particle as the macromolecule, considers Haworth's end-group method to relate to a minimum length and to be based on degraded triacetate, and formulates cellulose as a chain or thread of at least 2,000 glucose units with an end group which would give rise to tetramethyl glucose (I). It is true that Hess (15) has failed to detect this group, but with a molecule of such length this is hardly surprising and it may even be that the end group of native cellulose may have another structure.



I. Cellulose ($X = 100-200$ glucose units, Haworth;
 $X = 2000$ units, Standing)

Haworth (16) considers that native cellulose is most probably a molecular aggregate consisting of a much larger physical molecule than 200 units, such chains being joined by covalent bonds or by coordination end to end. Further, there may be forces which affect molecular aggregation laterally between adjacent chains. This is little more than an *ad hoc* explanation and appears untenable in the face of the evidence of degradation.

Neale (17) has summarised the difficulties experienced in estimating the reducing sugar group in cellulose in the small proportions which are to be estimated. Though a considerable measure of success has been obtained with starch, the method cannot be applied without ambiguity to purified cellulose, more particularly since such groups become modified by boiling with alkali producing carboxylic groups, which go into solution in the form of acids of low molecular weight. By adding the reducing power, measured by Richardson's

method, to the titration measured in a new way devised by Neale, a chain length of 1400 units is indicated. The method has been extended to hydrocelluloses with larger reducing power, for which the assumption that there is either an acidic or reducing group at one end of the chain is probably nearly true, and a comparison made between the average chain length indicated by this and by the physical methods. The results are summarised in Table II.

TABLE II
DEGREE OF POLYMERISATION OF MODIFIED CELLULOSES (NEALE)
(*Number of glucose residues*)

	From End Group	From Viscosity	Respective Breaking Load
Bleached yarn	1,400	1,260	100
Bleached cloth	740	720	100
Hydrocellulose i	210	210	50
Hydrocellulose ii	130	140	30
Hydrocellulose iii	92	100	20
Cellophane	85	105	..

A maximum molecular weight of cotton cellulose greater than 200,000 (1,200 units) is indicated by this method. When the cellulose is degraded, the tensile strength falls, and around 200 units it is only one-half of the original value, so that a fibre of such cellulose is of little value as a textile. The question whether the molecule of cellulose is of the order of 1,200 or 200 units thus assumes great importance for its use as a textile.

The observation made with the condensation products of hydroxydecanoic acid, studied by Carothers & Van Natta (18), is of interest in this connection since the molecular weight of such polymers can be well authenticated by titration of the end group. They found that fibres of useful tenacity could be obtained only from linear condensation products of a molecular weight greater than 10,000.

Whatever may be the original structure of cellulose, it is quite clear that it requires very little to make it break down into a long-chain compound, whilst further degradation results in a mixture of substances of varying chain length. It is the average chain length of these mixtures which determines the physical and chemical properties. Such chemical modification is produced by the action of acids

or of oxidising agents, the products formed being divided into hydro-celluloses and oxycelluloses. A systematic study of the chemically modified celluloses by Davidson (19) has been in progress for some time at the Shirley Institute, Manchester.

Hydrocelluloses.—The treatment of an original cellulose with acids brings about an increased reducing power as measured empirically by the weight (in grams) of copper reduced from the cupric to the cuprous state by 100 gm. of the material. The copper number of carefully purified cotton cellulose is only just significantly different from zero (0.05 or less). It rises as hydrolysis proceeds, whilst insoluble hydrocelluloses and soluble products of high reducing power are formed.

At the same time there is a fall in the tensile strength of the fibre which progressively weakens until it falls to a powder under very small stress, when the copper number has risen to about 5. Complete hydrolysis to glucose corresponds to a copper number of about 300, so that a relatively slight degree of hydrolysis has disastrous effects from the textile point of view.

A third factor that varies continuously with the acid treatment is the fluidity (the reciprocal of viscosity) measured by the rate of flow of the material dissolved in aqueous cuprammonium hydroxide. A definite relation exists between copper number, tensile strength, and fluidity irrespective of the conditions of the acid treatment.

This interdependence finds a simple explanation in terms of the molecular chain theory. The action of acids brings about hydrolysis at some of the glucosidic linkages, causing the formation of shorter chains, each of which bears a reducing group at one end. The attack occurs at points randomly distributed along the length of the chains so that whatever the condition of the original cellulose, it now becomes heterogeneous owing to chains splitting into fragments of various lengths. As hydrolysis by acid proceeds, there is a change in the direction of a continuously lower average chain length. The fluidity of a hydrocellulose is a function of this average chain length.

The fibre owes its tensile strength to the primary valency chains and to the additive effect of the lateral cohesive forces along these chains. Both tend to prevent slipping of one chain on another when a longitudinal force is applied. When the chains are shorter, their overlap is diminished so that slipping of the chains and consequently rupture of the fibre occurs more easily.

The modified celluloses dissolve more or less in cold concentrated

sodium hydroxide solutions, the solubility increasing continuously with progressive acid attack. This dissolution process is in reality a fractionation into longer- and shorter-chain molecules and "solubility" measures the proportion of the material that consists of chain molecules below a certain length. The reducing power of the soluble fraction is greater than that of the material as a whole.

Oxycelluloses.—The progressive action of an oxidising agent on cotton forms oxycellulose with progressively increasing fluidity and diminishing fibre strength, which can only be explained as due to a progressive shortening of chain molecules. There is hydrolysis of the glucoside linkages as well as oxidation of the terminal or of any other hydroxyl groups so that many simultaneous and consecutive reactions can take place. In confirmation of this, oxidation with dichromate which results in a copper number of five brings about a loss of tensile strength of only 20 per cent, whereas the same copper number resulting from acid attack corresponds to complete loss of strength.

When oxycelluloses are boiled with dilute alkalis, the fluidity is virtually unaffected and there is only a slight decrease in tensile strength. The chain molecules are attacked at the end bearing the reducing group, which is destroyed in the production of acidic groups. The long chains are shortened and the short chains decomposed into soluble products and removed; probably these two effects counterbalance each other and leave the fluidity unchanged. The shortening of the longer chains by relatively small amounts would not produce much effect on the tensile strength whilst the shortest chains, which are assumed to be removed, did not contribute to the strength.

The foregoing indicates that the behaviour of modified celluloses can be explained on the basis of the degradation of a long chain in the original cellulose which itself has necessarily had some chemical treatment in its preparation. The progressive hydrolysis by acids to hydrocellulose is due to the breaking of the glucosidic linkages, leading to a shortening of the chain. A method has been devised by Staudinger & Sorkin (20) for following the breakdown of a solid cellulose of about 1650 glucose units by means of viscosity measurements made on samples withdrawn at stated intervals. The stronger acids act more quickly than the weaker, but the degradation ceases after a period which corresponds to a rupture of about ten linkages and a polymerisation size of 150 to 200 units. Such figures are subject to certain corrections, but they give a rough indication of a primary

breakdown of the large molecule and add interest to the question whether each link in the long chain is equally susceptible to attack or whether there is first a tendency for a breakdown in the middle or at certain specific points. Some evidence on this question is discussed under starch.

Cellulose fibres.—The opinion that the cell wall is formed from many ultramicroscopic molecular aggregates termed "micellae" is an old one due to Naegeli. These micellae correspond to the crystallites or microcrystals composing the fibre. They are extremely small in diameter in comparison with their length and appear to be held together in the fibril by a cementing material of a pectic nature. The X-ray diagrams indicate that the orientation of the crystallites is far from perfect, but the presence of other cell-wall constituents which are amorphous does not appreciably modify the diagram. Meyer (21) has proposed a new crystal model for cellulose. Hitherto it has been assumed that all the micelles run in the same direction but there is no valid proof of this and the behaviour of precipitated hydrated cellulose makes it highly improbable that all the chains are similarly orientated. It is conceived that alternate chains are arranged in opposite directions and it is claimed that this model is more in accordance with measurements than the older one (22) which is now abandoned. Mark & Meyer had regarded the unit cell of ramie cellulose to be monoclinic of dimensions, $a = 8.35 \text{ \AA}$; $b = 10.3 \text{ \AA}$; $c = 7.9 \text{ \AA}$. In the new model, there is no longer any need for deviations from the tetrahedral angle in the side chains and the recalculated distance of 2.6 \AA between hydroxyl groups of neighbouring chains agrees well with recent measurements by Bernal (23) on hydroxyl distances.

It has been suggested that the average size of the micelles is of the order 600 \AA by 50 \AA , and it may be calculated that within these limits sixty cellulose chains could be packed, each consisting of 100 to 120 glucose units, which is the length deduced from the chemical evidence by Haworth. If the physical evidence is accepted that the chains are longer, then the conception that the micelles are more or less perfect and structurally discontinuous with their neighbours must be abandoned. Astbury (23a) takes this view and thinks that the micelles are interlocked by shared molecular chains.

It remains for the future to develop methods whereby the structure of fibres and of the cell wall may be studied. Structural fibres will probably differ from the simple fibres of the seed hair like cotton. In this connection, Clark (24) finds that the crystalline condition

becomes more perfect as the fibre approaches maturity, the arrangement of the chains being at random in the young fibre and orientation becoming visible only after thirty days.

To sum up, we can say with Sir William Bragg that chemical analysis and X-ray examination give a satisfactory picture of the cellulose chain-like molecule and some information also of the details of the molecular assemblages. Information is next wanted respecting the larger groups and the fibril formation on which the fibre properties obviously depend. If the fibre belongs to a living organism, change with time may be synonymous with growth.

There is no evidence that an ideal pure cellulose would be homogeneous; indeed, in textile research circles the opposite view prevails. In short, cellulose may be regarded as a chain-frequency distribution and the idea of one chain length must be held to be untenable.

STARCH

Starch in the living cell is being formed continuously and is subject also to rapid reconversion into sugars. The proper understanding of these processes of synthesis and simplification is still withheld from us and requires a knowledge of the structure of starch. Equally any chemical formula for starch must take into account its behaviour in the cell as well as in the test tube.

There are thousands of papers about starch in which the experimental work is often excellent; the difficulty is to get a harmonious interpretation of all the facts. Is starch made up of one or several substances? What part do the minor constituents (phosphorus, silicon, fatty acids) play in determining the properties of native starches?

In the chemical sense the word starch lacks an exact meaning, so that when seeking to determine the molecular size it is necessary to be precise regarding the material which is actually put into the chemist's flask. The previous happenings to the material between the plant and the flask may have caused a simplification in molecular aggregation whether this be due to ordinary or special valency forces. We have to seek to distinguish between modified and unmodified starches. As far as possible chemists will require a structure for starch in terms of ordinary valency forces.

Starch yields glucose on complete hydrolysis, maltose when broken down by enzymes, and dextrans under other conditions. The

traditional view held by Emil Fischer is that it consists of a chain of finite length made up of numerous monosaccharide units joined by glucosidic bonds, leaving open the question of whether the chain is straight or branched, or arranged in spiral or even closed ring form.

The opposition view, advocated during the last twenty years, postulated elementary units of low molecular weight with the special property of aggregating to form large molecules of colloidal dimensions by means of residual auxiliary or co-ordinate valencies. The type of units suggested ranged from glucosan to trihexosan. Today this view is definitely in the background.

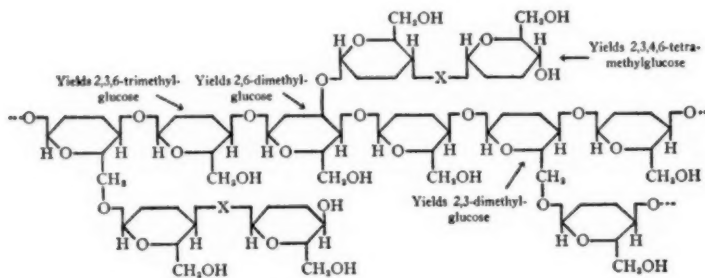
It is thought that a true chemical unit of starch does exist which the Birmingham school postulates as an open chain of α -glucopyranose units, approximately 24 to 30 in number, combined by 1,4-glucosidic linkages as in maltose. The conversion of starch to glucose is quantitative, i.e., the molecule contains no units terminating in a carboxylic acid.

Such a chain will have two different free ends, an aldehydic with reducing properties and a non-aldehydic with four free hydroxyl groups. Haworth (25, 43) developed the technique, (a) of complete methylation of starch, (b) of complete hydrolysis of the product, and (c) of the quantitative determination of small amounts of 2,3,4,6-tetramethylglucopyranose in the presence of a large quantity of 2,3,6-trimethylglucopyranose.

It is of interest to note the difficulty experienced in methylating starch up to the "trimethyl stage" which is equivalent to a content of 45.6 per cent methoxyl. Karrer (26), using methyl iodide and silver oxide, obtained 32.6 per cent; Schmid & Zentner (27) found only 21 per cent using diazomethane; Irvine & MacDonald (28) by means of dimethylsulphate obtained 61 per cent yield of a product with 27.2 per cent methoxyl; Freudenberg (29) obtained 38 per cent methoxyl by the same method. The most recent effort by Hess & Lung (30) has increased the methoxyl content by the dimethylsulphate process up to 42-43 per cent. Further treatment of this product with sodium in liquid ammonia followed by methyl iodide in the presence of anisol has yielded the completely trimethylated product. It may be that the difficulty in methylation is due to the formation initially of a dimethyl derivative.

Provided that none of these operations disrupts the starch molecule originally used, the Haworth technique gives a measure of the

number of glucose units in the chain, or alternately the average number of units in a mixture of chains of varying lengths. Criticism has turned on this point as it is claimed that some degradation does occur. It will be remembered that Haworth's methyl starch was obtained from an acetyl starch and it is considered that during the acetylation some simplification of the molecule takes place. The rupture of a small proportion only of the linkages in a long chain is sufficient to reduce the average length by a large amount. Thus, after hydrolysis of only 3 per cent of the linkages, a chain of 10,000 units becomes 301 chains of average length of 33.2 units and twenty chains of average length 500 become 319 chains of average length 31.4. A calculation on these lines by Richardson (37) indicates that 3 per cent hydrolysis of a mixture of chains aggregating 10,000 units of glucose in length would result in the formation of 310 to 350 chains of an average length of thirty glucose units. Inferentially Haworth has measured such average length rather than that of a specific macromolecule of starch. It may be that a chain of thirty glucose units represents a "chemical unit" with valency forces throughout and that several of these, more or less firmly joined together, form the mixture of chains of varying lengths postulated above. Incidentally the same unbranched chain, 26 to 30 glucose units long, has been obtained from starches of markedly different physical properties, e.g., by Haworth from potato, maize, or waxy maize and by Hassid & Dore (31) from canna.



II. Starch (Staudinger, X = 20 glucose units)

Staudinger (32) puts forward a formula (II) for starch which seeks to reconcile the very long chain view with Haworth's shorter

one. He pictures a central chain to which is attached a lateral chain at every fourth glucose unit through the CH_2OH side chain. In addition, lateral chains are joined to other glucose units through the hydroxyl attached to C_3 . These lateral straight chains are credibly of the length found by Haworth. Such a formula demands the identification of 2,3- and 2,6-dimethyl-, 2,3,6-trimethyl- and 2,3,4,6-tetramethyl-glucose from methylated starch.

The formula proposed is at least suggestive and will be provocative of further work. The difference between cellulose and starch as we know them in the so-called pure state is so great that it is difficult to believe that they differ only in chain length. It is easier to postulate that their secondary units are moderately long chains of some stability, and therefore surviving after mild chemical treatment, which are arranged according to an architectural plan which is essentially different. Whereas cellulose is threadlike, starch is an expanding substance capable of giving rise to the most fantastic shapes of grains in plants.

Another approach to the problem is made by Hanes (33) through a consideration of the action of the amylases on starch. The outstanding fact is that so far as susceptibility to attack by enzymes is concerned, the molecular chain structure is evidently not uniform over its entire length. The picture of enzyme attack on a macromolecule of thirty glucose units postulates that (a) the liquefying amylase breaks down bonds uniting several macromolecules, there is a cleavage of phosphorus, and the aldehydic groups become reducing; (b) β -amylase acts on the starch macromolecule from the non-aldehydic end and removes one molecule of maltose after another until it encounters a resistant residue of about twelve glucose units corresponding to the observed conversion of 60 per cent of the substrate into maltose; (c) α -amylase acting from the same end removes three units of a reducing dextrin containing six units of glucose and leaves the same resistant amyloextrin as before.

This limit dextrin, variously termed " α -amyloextrin" and "erythroggranulose," has been subjected to end-group analysis by Haworth, Hirst, Kitchen & Peat (34). The methylated dextrin was prepared by three different methods and gave figures indicating a chain of about twelve glucose units.

Myrbäck & Ahlborg (35) consider that the dextrans split off by β -amylase and by ptyalin originate from portions of the starch molecule lying between chain branching points or points where a phosphate

group occurs. They find that the phosphorus-containing portion of a starch or dextrin molecule is the most resistant to hydrolysis either by acids or by amylase.

It is widely accepted that starch can be divided into two fractions, amylose, which is water-soluble and "retrogrades" on concentration to form an insoluble precipitate, and amylopectin, a mucilaginous substance with the characteristic paste-forming properties. Their relative proportions vary considerably (36). Richardson (37) suggests that if unmodified starches contain a mixture of chains of varying lengths, the shorter, more easily soluble chains constitute the amylose fraction and the longer chains make up the amylopectin.

In this connection Reich & Damansky (38) find that acylation of potato starch produces a mixture of a diacetate (82 per cent) and a triacetate (16 per cent). These were separable quantitatively by solution of the triacetate in chloroform. The diacetate yields when hydrolysed a substance with the properties of natural starch called "amylogene." The triacetate gives rise to another substance, "amylon." When the diacetate is "acetolysed" the triacetate is formed and so amylogene becomes amylon. Amylopectin consists of amylogene with more or less amylon. Amylose is quantitatively the same as amylon. The amylon in starch is formed during its preparation, the transformation being brought about even by hot water; it consists in the liberation of one extra hydroxyl group, which can be esterified, for each glucose unit.

It can be postulated that in amylogene the chain of glucoses is compressed by folding so that one hydroxyl in each pair of units engages with that above and below it. Such a structure rather emphasises the maltose unit and the splitting off of successive maltose units by enzymes. When this internal link between hydroxyls is broken, the molecule is stretched in some way analogous to the well-known conception of coiled and uncoiled keratin. It has been established for the proteins that when a chain is coiled, the side-chain reactions are more and more satisfied by interactions in the molecule itself.

If the chain molecules have a finite length, starch will have a reducing power which will be less as the chain becomes longer, and an accurate determination of this group will allow a calculation to be made of the number of units in the chain. Haworth & Percival (39) claimed that their materials did not reduce Fehling's solution, but Richardson (37) found that unmodified starches definitely have re-

ducing powers ranging from 2.8 to 8.9 on a scale in which maltose = 2055. The evidence suggests that the reducing properties are associated with starch itself and were not due to impurities. Such figures correspond to average chain lengths of from 1470 to 460 glucose units, figures of an order of magnitude some fifty to twenty times that obtained by Haworth's methylation method (43). Osmotic pressure measurements (36) indicate a molecular weight corresponding to 1230 units, while sedimentation velocity in the ultracentrifuge (40) indicates 5550 units.

Soluble starch, sometimes considered to differ from natural starch only in its state of aggregation, shows, however it is made, a definite increase in reducing power compared with the parent substance, which is clear evidence that soluble starches consist of chains of shorter average length.

If all the linkages in a starch-chain molecule were equally susceptible to hydrolysis, the attack by hydrolytic agents other than enzymes might reasonably be expected to take place at random points along the chain so that the rate of increase in the number of reducing groups should be proportional to the number of unhydrolysed linkages present. This is not altogether the fact with a starch paste, indeed there is some evidence that the rate is slightly more rapid than would be predicted by the kinetic theory. It has been suggested that this is due to the more rapid hydrolysis of the shorter chain molecules.

Whatever may be established as the structure of the starch molecule, biochemists will demand that it gives some explanation of the ready transformation in the plant of starch into cane sugar, that is into glucopyranose and fructofuranose, since the conversion of starch into maltose is unknown in the ordinary metabolism of the green plant.

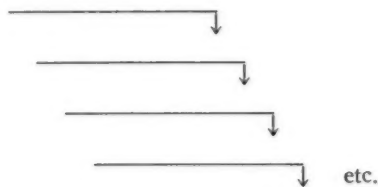
The manner of the synthesis of starch is also a problem. Does the synthesis come about as the result of the orientation and joining together of some thirty unit glucose molecules against a template, which may be pictured as an enzyme, to form the chemical starch conceived by Haworth which is later further associated till it makes a larger compound or mixture of such larger compounds? Alternatively, are the glucose units joined together continuously, one after the other, in the manner pictured in other branches of chemistry in the synthesis of large molecules (42)? Or do formaldehyde units undergo condensation to starch direct without passing through cane sugar?

OTHER POLYSACCHARIDES

A number of polysaccharides other than cellulose and starch are today the subject of intensive work. In only a few instances has the chain length been determined, interest centering chiefly on their constituent sugars and the nature of their attachment each to the other. It is already clear that here, as always in nature, there is infinite structural variety so that many different stereoisomeric substances are formed from a few materials. As experimental technique improves many more will be discovered.

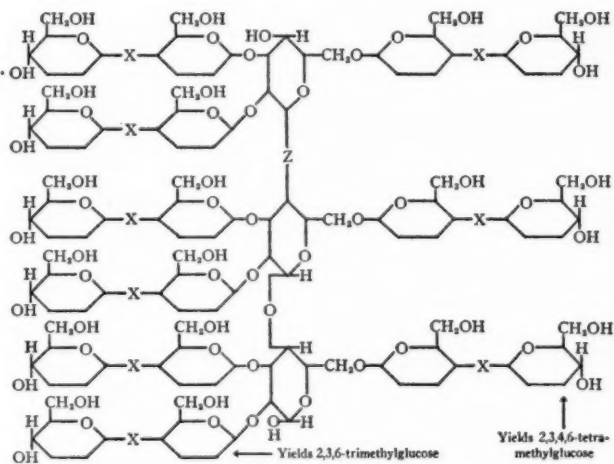
Glycogen.—This reserve carbohydrate of the animal cell is of a powdery nature and is considered by Haworth (40, 43) to have an average minimum chain length of twelve to eighteen glucose units. The analysis of rabbit-liver glycogen (44) indicates an average chain length of eighteen α -glucopyranose units. Fish-liver glycogen has twelve units, that of *Mytilus edulis* (45) eighteen units. After methylation and hydrolysis, all yield about equivalent amounts of di- and tetramethylglucose. Haworth suggests that the glucose chains are themselves joined by a type of union which links the reducing end of one chain with an hydroxyl of an adjoining chain present in a non-terminal glucose residue. Such glucose residue would contain only two exposed or free hydroxyl groups which would appear therefore as dimethylglucose on methylation and hydrolysis.

Diagrammatically this conception may be expressed as



each line representing a chain of twelve to eighteen units and the arrow the point of attachment. It must be a matter of conjecture what kind of a linkage takes place. A special case of this formulation would be a continuous main chain of glucopyranose units in the form of a loop having other residues attached at intervals as side chains. It is stated that these molecules display little or no tendency towards molecular aggregation.

Staudinger & Husemann (46) conclude from their own and Haworth's observations that glycogen consists of a central chain of glycosidically bound units of glucose linked C_1 to C_4 , to which are attached at C_2 , C_3 , and C_6 of each unit similar side chains of from twelve to eighteen units in length (III). A substance of this formula, with a main chain of thirty to forty units and with side chains in aggregate of the same length, will constitute a macromolecule approximating to a ball made up of some 2,400 units in all. Under this theory the chain lengths measured by Haworth are the side chains detached from a degraded glycogen.

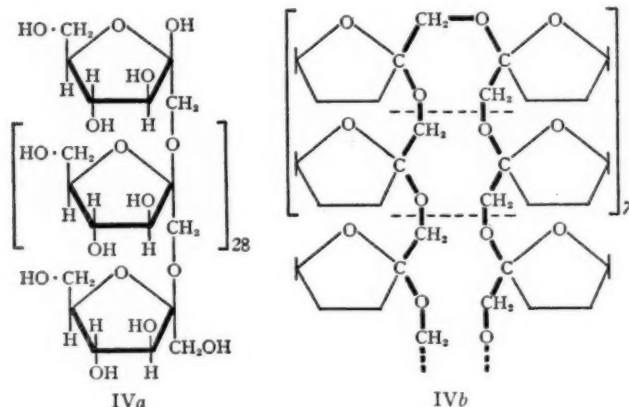


III. Glycogen (Staudinger, X = 12-18 glucose units:
Z = up to 100 units)

The galactogen (47) from the snail yields equal amounts of tetra- and dimethylgalactose on hydrolysis of its methyl derivative. It is thus quite differently constituted from glycogen.

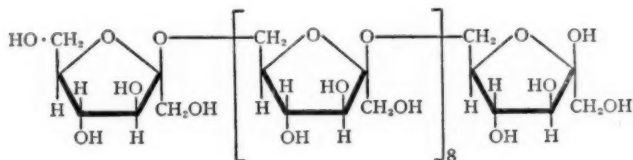
Fructose polysaccharides.—Inulin, according to the end-group method of assay, is composed of a chain of thirty fructofuranose units united by 1,2-junctions (IVa). This molecular size is confirmed by the alternative physical methods of measurement. The fact that it breaks down to the 1,2-difructofuranose anhydride has led Haworth

(48, 49) to formulate it so that a simple cleavage along the dotted lines will liberate pairs of fructofuranose units (IVb).



Fructose

Levan (50), produced by the action of *B. mesentericus* on sucrose, and possibly identical with a water-soluble polysaccharide (51) present in the leaves of certain grasses, has a chain length of ten fructofuranose units joined through C₂ and C₆ (V).



In addition to inulin which has a large molecule, several fructosans have been newly described, most of which give some quantity of dimethylfructose when their methyl derivative is hydrolysed (Table III).

Schlubach favours a large ring structure rather than a long chain for these compounds. Irisin (52), the polyfructose present in the rhizomes of *Iris* species, probably has as polymerising unit a difructofuranose in which the junction is between C₂ and C₄. Graminin (53, 54) is remarkable in that the methylated derivative is hydrolysed to approximately equal proportions of tetra-, tri-, and dimethylfructoses, which indicates that it may consist of a nucleus of four fructose residues with side chains. In sinistrin (55) it is suggested there are fifteen fructose residues. In tritacin, which is highly stable, Schlubach & Peitzner (56) assume a closed ring of fructose residues, which is a multiple of 7, viz., either 14 or 21.

TABLE III

RATIO OF DI-, TRI-, AND TETRAMETHYLFRUCTOSE FORMED ON HYDROLYSIS

Fructosan	Tetra	Tri	Di
Irisin	1	0	1
Graminin	1	1	1
	or 2	1	2
Sinistrin	1	3	1
Tritacin	3	1	3

Yet another fructosan, namely asparagosin, from the tubers of *Asparagus officinalis*, is described by Murakami (57) as made up of only seven fructose units, but Schlubach (58) attributes ten units to it corresponding to the determined molecular weight. The proportions of tetra-, tri-, and dimethylfructoses obtained from it are 1:8:1. Another substance, asphodelin (59), has the proportions 1:5:1. One of these is said to be glucose, which is probably derived from an impurity.

Hemicelluloses.—The hemicelluloses are unfortunately named since they have no relation to cellulose. Obtained by extraction with dilute alkali and precipitation with dilute acid, they are more susceptible to hydrolysis than cellulose.

In addition to hexose and pentose, they always contain sugar acids of the uronic type. They may be divided into a glucose series made up of *d*-glucose, *d*-xylose, and *d*-glucuronic acid and a galactose series made up of *d*-galactose, *l*-arabinose, and *d*-galacturonic acid. Whereas in the first group xylose usually exceeds glucose in amount, it is more customary to find galactose in excess of arabinose. Man-

nose is known as a hemicellulose constituent but the presence of the uronic acid is doubtful and the corresponding pentose, *d*-lyxose, has not been found in nature. In the present state of knowledge, bearing in mind the difficulty of isolating them in a condition of purity, it is not advisable to do more than indicate what is known as to their structure and constituents.

Mannose polysaccharides.—Mannan A (60), from ivory nut, is stated to consist of a chain of some eighty mannopyranose units linked through positions 1 and 4. The similar mannan from salep (61) has been assigned a chain length of sixty units. It should be noted that this mannan is fairly soluble in water, whereas the ivory-nut mannan is insoluble and its molecule must in fact be much larger than eighty units.

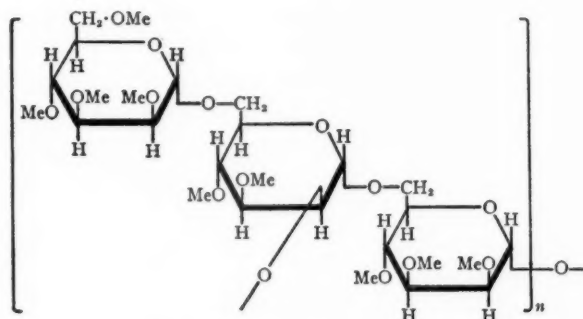
A much simpler mannan termed "mannocarolose" (62), produced when *Penicillium Charlesii* G. Smith is grown on Czapek-Dox-glucose solution, is deemed to be a chain of nine mannopyranose units joined together through the 1,6 positions by α -glycosidic links. Another polysaccharide is produced at the same time, namely galactocarolose (63), which apparently has a minimum chain length of nine to ten units of β -galactofuranose linked through the 1,5 positions. This occurrence of a furanose sugar is most interesting.

Another example is afforded by a polysaccharide, β -pachyman (64), obtained from a fungus, *Bukuryō*, which grows usually on the root of the pine, said to be composed of glucofuranose. The furanose units are thought to be mutually linked at the first and fifth carbon atoms and to be alternately α and β . The molecular weight of β -pachyman is smaller than that of starch. There is indication of the formation of a disaccharide during hydrolysis.

A third mannan obtained from yeast gum is quite remarkable. Haworth, Hirst & Isherwood (65) find that the methylated molecule yields on hydrolysis three products in equimolecular proportions, viz., 2,3,4,6-tetra-, 2,3,4-tri-, 3,4-dimethylmannopyranoside, and is therefore made up of three types of residue. The size of the molecule is probably large. One is attached by its reducing group to another mannose residue and must form a terminated side chain; a second is attached at positions C_1 and C_6 to other mannose residues, whereas a third is attached to other mannose residues at positions C_1 , C_2 , and C_6 .

The simplest formulae to express these findings comprise a chain of α -mannopyranose units linked through the 1,6 positions with a

side chain consisting of a mannose unit joined at C₂ of every alternate residue (VI).



VI. Methylated yeast gum mannan

Recent literature contains references to mannans of Japanese saleps (66) and the tubers of *Bletilla striata*.

Galactose polysaccharides.—A preparation from bean, vine, and other leaves yields only galactose on hydrolysis (67). The galactoaraban (68, 69) of various larch woods has a minimum molecule of six galactose and one arabinose units. Another galactoaraban (70) from peanut seeds is stated to be composed of a repeating triose molecule consisting of one galactose and two arabinose units. A proportion of uronic acid may or may not be an impurity.

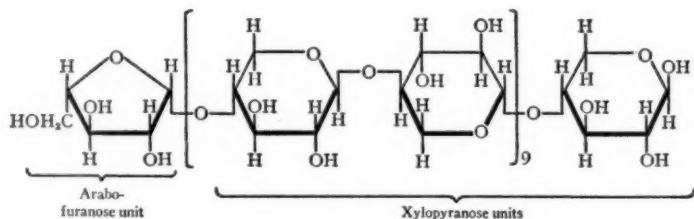
Agar-agar, so far as the main carbohydrate portion is concerned, is stated by Percival (71) to consist of β -galactopyranose units linked at position 1,3, since the main product of hydrolysis of the methylated agar-agar is 2,4,6-trimethylgalactose. It is not clear whether the units are present in the form of a six-membered ring or alternatively as a zigzag chain terminated by residues as yet undetermined. There is also the possibility that they may be linked by means of an intermediary ketose residue.

A most remarkable polysaccharide is varianose (72), synthesised by *Penicillium varians* G. Smith. It is regarded as a chain of six to eight galactopyranose units with 1,4 linkages, to which is attached at one end a glucopyranose unit and at the other (reducing) end a unit of either *l*-altrose or *d*-idose. It is probable that we are only at the beginning of our knowledge of this type of polysaccharide.

The gums and the mucilages contain a number of sugars, whilst there is evidence that both pyranose and furanose ring structures may be present. The sugars isolated on hydrolysis consist of four hexoses, two pentoses (arabinose and xylose), two methylpentoses (rhamnose and fucose), as well as uronic acids (glucuronic and galacturonic acid).

Hirst & Jones (73) state that gum arabic consists of glucuronic acid joined to C₆ of galactose, whereas damson gum contains glucuronic acid joined to mannose, the main chain being continued by two galactose residues. Further, it has two arabinose residues attached as side chains with a furanose ring structure. Wood gum contains an arabofuranose unit attached to a main chain of xylopyranose residues.

The xylan (74) from esparto grass has been prepared in a purer state and found to yield on hydrolysis 93 per cent of xylose together with 7 per cent of arabinose. It is highly probable that the chemical molecule consists of eighteen or nineteen xylopyranose units with 1,4 linkage, the chain being terminated at one end by an arabofuranose group (VII).



VII. Xylan

A substance of considerable interest in relation to the origin of hemicelluloses in wood is the so-called "starch" isolated by Campbell (75) from oakwood sap and oak leaves. This contains 14 per cent of uronic acid residues, 10 per cent of xylose, and 75 per cent of glucose and is characterised by giving a royal purple colour with iodine, finally becoming blue. It represents a starch of which one quarter of the molecule is undergoing transformation to uronic acid and pentose. From the leaf "starch," as much as 25 per cent of uronic acid was obtained. These compounds are possibly stages in the long-since-formulated theory of oxidation of the primary alcohol and decarboxylation of the acid leading from hexose to pentose.

Pectin.—The formula of pectin has hitherto been based on the work of Ehrlich (76), who regarded it as a complex in which four contiguous galacturonic acid units were combined with galactose and arabinose; three of the acid groups were methyl esters and there were also two acetic acid units. The six sugar units were grouped to form a large ring.

The systematic work of Schneider (77, 78) has now shown this conception to be incorrect; there is no acetic acid and the arabinose and galactose are impurities which can be removed. Pectin consists entirely of a long chain of galacturonic acid units joined 1,4, some of which are esterified with methyl alcohol.

The various plant pectins and technical products differ in molecular length, which probably has a relation to their thread and film-building and gelifying properties, in the degree of esterification and in the amount of impurities. Beet pectins are of smaller molecular length than fruit pectins, hence their inferior properties.

Immuno-polysaccharides.—Space permits only the shortest reference to these, particularly as a summary has been provided elsewhere (79). There are five recognised types, of which all but Type IV contain uronic acids. Types II, III, VIII contain only traces of nitrogen. The basic unit of Type I is a trisaccharide containing two molecules of *d*-galacturonic acid. Type III (80) yields on hydrolysis glucose (9.5 per cent) and an aldobionic acid (85 per cent) formed of glucose and glucuronic acid. This is thought to be 6-glucuronosido- β -glucose (81).

The polysaccharide of *B. dysenteriae*, studied by Morgan (82), has as basal unit four hexose molecules and one N-acetylaminohexose molecule, the unit being repeated six times in the molecule.

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THE CHEMISTRY OF THE ACYCLIC CONSTITUENTS OF NATURAL FATS AND OILS*

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Most of the investigations to be reviewed at this time deal with recent reports of the component acids and other constituents of fats, oils, and waxes. As usual, it has been found that the number of contributions on vegetable fats is much larger than that devoted to those of animal origin. The isolation and identification of minor constituents of fats is still receiving much attention and some interesting discoveries have been made.

PROGRESSIVE CHANGES IN NATURAL FATS

Hilditch & Lovern (1) made a general survey of progressive changes in the component acid mixtures present in natural fats. These run parallel in many respects with the evolutionary development of plants and animals. It was found that fats associated with the most primitive forms of life are the most complex in type and have a preponderance of unsaturated acids. Although this persists throughout the larger marine animals there is a distinct difference between the fats of fresh- and salt-water fishes. It is noteworthy that these unsaturated acids are present in smaller quantities in the fats of amphibians, reptiles, birds, and mammals. In mammals they occur only in very minute quantities or not at all. The authors state that the fats of land animals on the one hand, and of the endosperms and embryos of fruits of the larger plants on the other, exhibit broad specific resemblances, subject to generic or family differences according to their biological relationships. They have suggested that systematic classification of the natural fats should begin with the complex types of primitive flora and fauna and proceed from that point to amphibians, birds, and mammals, as well as to the more highly developed land flora.

COMPONENT GLYCERIDES

Hilditch & Stainsby (2) have made a very extensive investigation of cacao-butter glycerides. The methods used included those for com-

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ponent acid analysis of the whole fat and the fully saturated glycerides in the fat after partial and after complete hydrogenation. After partial fractionation of the glycerides of the original fat by crystallization from acetone, each fraction was examined by the same procedures as already mentioned. It was found that cacao butter contained the following percentages of glycerides: oleopalmitostearins 52, oleodistearins 19, steardioleins 12, palmitodioleins 9, oleodipalmitins 6, and palmitostearins 2. The authors concluded that β -palmito-oleostearin, β -oleodistearin, and β -oleodipalmitin are probably the predominating isomerides of these glyceride types.

VEGETABLE FATS

Steger & van Loon (3) investigated essang oil from the seeds of the West African tree, *Ricinodendron africanum* of the Euphorbiaceae. The seed kernels contained 48.8 per cent of oil having the following characteristics: n_{20}^D 1.5054, saponification value 193.1, iodine number 192, unsaponifiable matter 0.5 per cent, and thiocyanogen value 86.6. The percentages of fatty acids in the oil are as follows: oleic 16.0, linoleic 11.0, linolenic 10, elaeostearic 46.0, and saturated acids 10.0. The oil also contained 1.2 per cent of volatile matter. It is believed that this is the first case reported in which an oil contained both linolenic and elaeostearic acids.

Steger, van Loon & Smelt (4) examined the seeds and oil of *Ricinus zanzibarinus*. The seeds contained 74 per cent of kernels having 65.1 per cent of oil. Some of the more important characteristics reported for the oil are as follows: iodine number (Wijs) 88.4, saponification value 179.2, thiocyanogen value 82.0, acetyl value 145, and unsaponifiable matter 0.5 per cent. The mixed fat acids consisted approximately of 1.1 per cent of saturated acids, 6.6 per cent of linoleic, and 92.3 per cent of ricinoleic acid. The oil is much like ordinary castor oil from *Ricinus communis* in composition and properties, but it contains less saturated acids and somewhat more linoleic acid.

Branke & Gutt (5) reported that the seeds of *Xanthium strumarium* (Fabiaceae) contained about 40 per cent of oil having the following characteristics: d_{15}^{15} 0.9197; n_{20}^D 1.4757; acid value, 0.66; saponification value, 189.8; iodine number, 137.4; and unsaponifiable matter, 0.85 per cent. The composition of the insoluble fatty acid fraction was given as follows: palmitic 1.5 to 2.0, stearic

7 to 7.5, oleic 26.7, and linoleic 64.8 per cent. From this investigation the oil appears to be similar to that from sunflower seed.

Goldstein & Jenkins (6) by petroleum-ether extraction of dried poke root (*Phytolacca americana*) from North Carolina found that it contained 0.44 per cent of oil which gave the following characteristics: d_{25}^{25} , 0.9209; $[\alpha]_D^{26}$, $+13^\circ$; n_D^{26} , 1.4741; acid value, 71.97; saponification value, 139.4; ester value, 67.4; and iodine number, 69.14. It was found to be a complex mixture of glycerides, wax-like esters of fatty acids with a sterol, and a large quantity of free fatty acids. A sterol named phytolaccasterol ($C_{30}H_{50}O \cdot H_2O$; m.p. 169° – 170° ; $[\alpha]_D^{36}$, $+35^\circ$) and a sterol-like substance ($C_{23}H_{40}O$; m.p. 107° – 108° ; $[\alpha]_D^{20}$, $+70^\circ$) were isolated.

As the latter substance did not react either with acetic anhydride or acetyl chloride, the oxygen does not appear to be a part of a hydroxyl group. The percentages of fatty acids isolated from the oil were as follows: palmitic 8.63, margaric 4.19, oxymyristic 0.72, and arachidic 5.91. Presumably the margaric acid ($C_{17}H_{34}O_2$) reported by these authors was a mixture, as in previous cases in which it has been claimed to be present. Oleic acid was identified but not determined.

Gupta & Dutt (7) found that the seeds from the fruits of the Indian medicinal plant, *Solanum xanthocarpum*, contained 19 per cent of a greenish yellow oil which gave the following characteristics: iodine number 124.3, saponification value 182.5, acid value 77.8, and unsaponifiable matter 1.2 per cent. The oil was reported to contain 42.93 per cent of oleic acid, 36.18 per cent of linoleic acid, 5.37 per cent of palmitic acid, 9.77 per cent of stearic acid, and 0.35 per cent of acids of higher molecular weight.

Delvaux (8) examined plum pit and beechnut oils. The kernels from plum pits contained 42 per cent of oil which gave an iodine number of 100.3, a thiocyanogen value of 81.2, and which contained 0.4 per cent of unsaponifiable matter. The calculated percentages of component fatty acids in the oil were as follows: oleic 68.9, linoleic 21.0, and saturated acids 5.7. Dried beechnut kernels were found to contain 42.2 per cent of oil which gave an iodine number of 111.9, a thiocyanogen value of 79.2, and a hexabromide number of 3.37. The unsaponifiable matter amounted to 0.27 per cent. The calculated percentages of component fatty acids in the oil were as follows: oleic 48.4, linoleic 33.2, linolenic 2.8, and saturated acids 11.5.

Mikhelson (9) reported that the dried seeds of *Ailanthus glandu-*

losa, which grows in middle Asia, contain 35.9 per cent of oil, having an iodine number of 121.4, and an ester value of 187.3. The insoluble fatty acids consisted of 80 per cent of oleic acid, 5 to 6 per cent of linoleic acid, 9.4 per cent of palmitic acid, and 2.4 per cent of stearic acid.

Cruz & West (10) reported that oil of the Philippine physic nut (*Jatropha curcas*), which gave an iodine number (Hanus) of 94.8 and a saponification value of 192.4, contained the following percentages of fatty acids as glycerides: oleic 61.86, linoleic 18.65, myristic 0.45, palmitic 11.84, stearic 5.07, and arachidic acid 0.26.

Cruz & West (11) examined a sample of Philippine tobacco-seed oil, which gave a saponification value of 190.5, and an iodine number (Hanus) of 135.8, and contained 1.41 per cent of unsaponifiable matter, 9.99 per cent of saturated, and 82.87 per cent of unsaturated acids. The percentages of fatty acids in terms of glycerides are as follows: oleic 26.37, linoleic 60.23, myristic 0.05, palmitic 7.03, stearic 3.04, and arachidic acid 0.34.

Sullivan & Bailey (12) made an extensive investigation of the oil extracted from germ material of Marquis spring wheat separated by means of the usual milling procedure. The more important characteristics of the oil were as follows: saponification value 184, iodine number (Rosenmund-Kuhnhen) 125, thiocyanogen value 84.7, hexabromide value 2.28, and unsaponifiable matter 4.00 per cent. The saturated acids, which amounted to 16 per cent of the oil, consisted of palmitic, stearic, and lignoceric acids. Over 70 per cent of this fraction was palmitic acid. The unsaturated fatty acids were estimated to contain 28.14 per cent of oleic acid, 52.31 per cent of linoleic acid, and 3.55 per cent of linolenic acid. Data are also given for the bromination and the alkaline permanganate oxidation experiments with the unsaturated acids. The authors (13) reported that the unsaponifiable fraction consisted approximately of 70 per cent of sterols, of which 56 per cent occurred in the free state. In addition to isomeric sitosterols and dihydrositosterol, evidence was obtained for the presence of another type of sterol having at least two double bonds. The non-sterol fraction of the unsaponifiable matter appeared to be a mixture of polyene hydrocarbons and one or more alcohols.

Kaufmann & Baltes (14) applied their diene (maleic anhydride) value to two different samples of tung oil, obtaining values (a) 68 and (b) 70. They estimated that these oils contained (a) 75.4 and (b) 76.7 per cent of elaeostearic acid. Calculations with the iodine

and thiocyanogen values of the oils gave much higher results. The authors claimed that this was due to the presence of linoleic acid. They reported that oil (a) contained 9.7 and oil (b) 6.8 per cent of this acid which apparently heretofore was not known to be present. In a similar manner, oiticica oil was examined. It gave a diene value of 60.8, which indicated that it contained 70 per cent of licanic acid and 15.2 per cent of non-conjugated unsaturated acids. It remains to be determined whether this oil also contains linoleic acid.

McKinney & Jamieson (15) examined a sample of Brazilian oiticica oil expressed from the seeds of *Licania rigida*. The characteristics of the oil were as follows: saponification value 192.6, iodine number (calc.) 218.0, thiocyanogen value 76.2, unsaponifiable matter 0.57 per cent, and n_{D}^{25} 1.5145. The percentages of fatty acids in the oil were reported as follows: oleic 5.9, licanic 78.2, and saturated acids 10.7. The percentage of licanic acid was calculated on the assumption that oleic acid was the only other unsaturated acid present. However, Morrell & Davis (16) have shown that at least 4.7 per cent of elaeostearic is present, and this is in agreement with Kappelmeier (17).

Jamieson & McKinney (18) have made the first extensive investigation of the oil of the black walnut (*Juglans nigra*). The characteristics of the oil were as follows: saponification value 193.5, iodine number (Hanus) 135.1, thiocyanogen value 86.0, unsaponifiable matter 0.42 per cent, and n_{D}^{25} 1.4730. Another sample of the oil gave an iodine number of 140.5. The percentages of fatty acids in the oil were as follows: oleic 34.1, linoleic 46.8, linolenic 7.2, myristic 0.43, palmitic 3.29, stearic 1.77, and arachidic 0.04. The iodine number indicates that it belongs to the lower range of drying oils.

The oil expressed from seed of Java kapok (*Ceiba pentandra*) was investigated by Jamieson & McKinney (19). Its characteristics were as follows: iodine number (Hanus) 96, saponification value 190.7, acid value 3.7, acetyl value (Andre-Cook) 12.9, unsaponifiable matter 0.80 per cent, and n_{D}^{25} 1.4696. This pale yellow oil was found to contain the following percentages of fatty acids: oleic 43.0, linoleic 31.3, palmitic 9.77, stearic 8.00, arachidic 1.19, and lignoceric 0.04. During recent years large quantities of the seed imported from Java have been pressed on the Pacific Coast, and the oil after refining has been used in this country for edible purposes.

Smit & van Loon (20) examined the oil from the seeds of *Telfairia pedata* (Curcubitaceae) which had the following characteristics:

iodine number (Wijs) 88.2, thiocyanogen value 52.7, saponification value 193.7, unsaponifiable matter 0.9 per cent, and volatile matter 3.3 per cent. The percentages of component fatty acids were as follows: oleic 11.4, linoleic 32.6, linolenic acid 5.0, palmitic 24.3, and stearic 18.2. In connection with this investigation, it is interesting to note that the telfairic acid reported by Thoms (21) as an isomer of ordinary linoleic acid has been shown to be non-existent in this oil by the above investigators and also by Goodale & Haworth (22).

Hilditch & Ichaporia (23) determined the component fatty acids for three Solanaceae seed oils by the usual fractional distillation of the methyl esters of the "solid" and "liquid" acids obtained by the lead-salt alcohol separations. The percentages of component fatty acids reported for each oil were as follows: *Datura stramonium* oil—1.5 of myristic, 10.8 of palmitic, 1.2 of stearic, 33.1 of oleic, and 53.6 of linoleic acid; *Atropa belladonna* oil—5.9 of palmitic, 1.8 of stearic, 25.5 of oleic, and 53.6 of linoleic acid; *Hyoscyamus niger* oil—6.5 of palmitic, 0.4 of stearic, 11.1 of oleic, and 82.0 of linoleic acid. The association of a small quantity of saturated acids with considerable oleic and large amounts of linoleic acid appears to be characteristic for all the Solanaceae seed oils which have so far been examined. A further investigation of the *Datura* oil showed the "even distribution" of the fatty acids between the glycerol molecules, showing that the latter are assembled in the characteristic manner of most of the seed fats which have been investigated.

Täufel, Thaler & Schreyegg (24) examined a sample of yeast fat separated from "Cerolin" (an alcoholic extract of brewers' yeast) and stated that it contained the following percentages of acids: volatile acids 5.2, palmitic 9.5, stearic 5.9, oleic 47.6, and linoleic 2.9. The volatile acids consisted chiefly of valeric acid (25, 26). The unsaponifiable matter, which amounted to 19.6 per cent, consisted of 3.3 per cent of sterols and 16.3 per cent of the hydrocarbon, squalene.

The same investigators (27) extracted the fat from a mold (*Citromyces Sp.*) with petroleum ether, and found it to consist of 9.9 per cent of unsaponifiable matter, 5.8 of palmitic, 10.0 of stearic, 34.4 of oleic, and 34.4 of linoleic acid. It was concluded that the absence of fatty acids between C_8 and C_{14} is an indirect indication that the production of higher methyl ketones by molds from fat acids is not a normal process.

Puntambekar & Varma (28) have reported the results of their examination of the acorn kernels and the kernel oil of *Quercus incana*.

The kernels contained the following percentages of constituents: moisture 12.2, oil 16.0, ash 1.4, proteins 3.0, tannins 4.2, cellulose 1.4, and carbohydrates 59.5. An analysis of the acorn cups is also given. The characteristics of the oil are as follows: d_{25}^{25} , 0.9081, n_D^{20} , 1.4576, saponification value 192.2, iodine number (Hanus) 81.5, unsaponifiable matter 0.8 per cent, saturated acids 18.0 per cent, and unsaturated acids 82.0 per cent.

Heretofore, comparatively little attention has been given to the examination of acorn oils, and the recorded data were very meager. Hutchins (29) examined both the crude and refined oils from acorns of the pin oak (*Quercus palustris*) which grows in South Carolina. The acorns contained 13.40 per cent of oil and 24.08 per cent of moisture. The characteristics of the crude oil were as follows: iodine number (Wijs) 99.4, saponification value 192.8, thiocyanogen value 73.7, acetyl value 7, and unsaponifiable matter 1.11 per cent. It was calculated that the oil contained the following percentages of acids as glycerides: oleic 58, linoleic 27, and saturated acids 14.5. Although the oil had an intense yellow color it was found that it could be bleached more readily than palm oil. Also it was easy to refine and deodorize the oil, giving a product of high quality suitable for edible use.

Grandilla passion-fruit seed (30) from Kenya contained 8.5 per cent of moisture and 22.4 per cent of oil having the following characteristics: d_{12}^{15} , 0.9261; n_D^{20} , 1.4761; acid value, 0.3; saponification value, 190.9; iodine number (Wijs), 141.2; and unsaponifiable matter, 0.8 per cent.

Moss (31) found that dried fruit of the banana contained from 0.7 to 0.8 per cent of oil which contained 14.4 per cent of unsaponifiable matter. The oil gave a refractive index at 40° of 1.4648 and an iodine number of 82, whereas the fatty acids having a mean molecular weight of 286 gave an iodine number of 90.

Lehrman & Kabat (32) found that banana starch contained 0.20 per cent of fatty acids giving an iodine number of 59.4. Repeated tests failed to show the presence of any glycerides. The fatty acids were found to consist of a mixture of palmitic, oleic, linoleic, and linolenic acids together with a very small quantity of phytosterol. This is the first time that a phytosterol has been found combined in a starch.

Jamieson & McKinney (33) examined a sample of oil which they expressed from kernels of Philippine lumbang (*Aleurites moluccana*).

The oil gave an iodine number (Hanus) of 151.7, a thiocyanogen value of 97.1, a saponification value of 190.8, and contained 8.39 per cent of saturated acids, 86.61 per cent of unsaturated acids, and 0.30 per cent of unsaponifiable matter. The percentages of fatty acids in the oil were as follows: oleic 26.23, linoleic 36.62, linolenic 20.76, palmitic 4.38, stearic 3.93, and arachidic 0.08. In contrast to the seed oils of the other *Aleurites* species, no elaeostearic acid could be detected.

Paget (34) has reported the results of his investigation of the liquid acid fraction of sapucainha oil from the seed of *Carpotroche brasiliensis*. It was estimated that between 65 to 70 per cent of the acids of the oil consisted of chaulmoogric, hydnocarpic, and palmitic acids, and that there was about 4 per cent of oleic acid, 9 per cent of dehydrochaulmoogric acid ($C_{18}H_{30}O_2$), and 4 per cent of keto-chaulmoogric acid ($C_{18}H_{30}O_3$, m.p. 116°) and ketohydnocarpic acid ($C_{16}H_{26}O_3$, m.p. 108°). Andre & Jonatte (35) first isolated and described dehydrochaulmoogric acid, which they named gorlic acid, having found it in the oil of gorli seed (*Oncoba echinata*). They stated that this acid is, doubtless, a constituent of all other optically active oils of the chaulmoogra group.

Kaufmann & Baltes (36) have developed a procedure for the determination of the diene value which is based on heating 0.10 to 0.15 gm. of oil with 10 cc. of a one per cent acetone solution of maleic anhydride for twenty hours at 100° in a sealed tube, adding water, and finally titrating the excess of maleic acid with 0.1 N alkali. Shortly afterwards, Ellis & Jones (37) suggested a quicker procedure in which 3 gm. of the sample to be tested is heated with 25 cc. of a six per cent toluene solution of maleic anhydride for three hours using a special reflux condenser, finally extracting the cool solution with water, and titrating the latter with normal alkali. Both procedures, when applied to tri- β -elaeostearin, gave values very close to the theoretical, which is 87.2. Kaufmann & Baltes, as well as Ellis & Jones, applied the methods to samples of linseed oil and found diene values ranging from five to seven. However, in a more recent communication by Kaufmann, Baltes & Bütter (38), describing their iodometric method for the determination of the diene value, they gave results for linseed-oil fatty acids ranging from 0.34 to 0.67. Application of the alkalimetric procedure to a sample of linseed oil (1.09 to 1.37) gave a diene value of 7.4. Of the twelve oils tested they found that only cacao-butter and palm-kernel oil gave no diene values, as was also

the case with triolein. The reason that the mixed fatty acids (for example, in the case of linseed and soybean oils) give very small diene values, as compared with those given by the original oils, remains to be determined. The experiments indicate that the diene values given by the oils tested, other than those of tung and oiticica, are not due to glycerides containing acids with conjugated double bonds.

Pelikan & Mikusch (39) recently reported on their investigation of the Kaufmann and the Ellis-Jones procedures. With certain substances they found that the Ellis-Jones method gives results which are too high and, further, that they appear to depend somewhat upon the weight of the sample taken for analysis. They stated that they found no indications to support the statement of Ellis & Jones that the Kaufmann method is unreliable.

Meinel (40) has developed a qualitative test for the presence of conjugated double bonds. Briefly, the method is based upon adding to the oil to be tested enough of a methyl alcohol solution of bromine to exactly saturate one double linkage. This reaction leads to the formation of brom-methoxy compounds which give off bromine easily only in the presence of conjugated double bonds. Upon the addition of silver thiocyanate the liberated bromine reacts with it, forming hydrogen thiocyanate which is detected by the red color it gives with a ferric salt. When applied to linseed oil, only a very faint pink color developed after the test had stood for fifteen minutes. A similar test made recently in the reviewer's laboratory failed to show any color until after the reaction mixture had stood about half an hour, and then but very faintly, whereas a solution containing only two per cent of tung oil immediately gave the characteristic color.

ANIMAL FATS

Commercial chrysalis oil and that prepared from several varieties of *Bombyx mori* were examined by Bergmann (41). The commercial oil was found to contain the following percentages of component fatty acids: palmitic 20, stearic 4, palmitoleic 2, oleic 35, linoleic 12, and linolenic 28. Besides these there was less than one per cent of saturated and between one and two per cent of unsaturated acids having more than eighteen carbons. The commercial oil was found to contain a considerable quantity of the glyceryl-1,3-dipalmitate. The oil from the tent moth, *Malacosoma americana*, had a composition very similar to that of the chrysalis oil.

Parry & Smith (42) have investigated the fat acids of ox-blood

lipids and reported the following percentages: palmitic 10, stearic 18, higher saturated acids 3, oleic 20, linoleic 6, C_{20} unsaturated acids 33, and C_{22} unsaturated acids 10.

Riemenschneider & Ellis (43) made an extensive investigation of goat-milk fat, using a large composite sample from a herd of 24 Saanen and Toggenberg does. The percentages of the component fatty acids reported were as follows: butyric 2.1, caproic 1.9, caprylic 2.7, capric 7.9, lauric 3.5, myristic 10.2, palmitic 28.7, stearic 8.1, lignoceric 0.4, decenoic 0.2, tetradecenoic 0.4, hexadecenoic 2.1, oleic 31.2, arachidonic and C_{22} acids 0.7. These results show general agreement with those previously published, except for the absence of linoleic acid and the presence of small percentages of decenoic, tetradecenoic, and hexadecenoic acids.

Hilditch (44) discusses the minor component fat acids of milk fats and gives much data in regard to the quantity of these acids in milk fats from different localities and for different seasons of the year. Also attention is given to the effect of feeding cod-liver and other oils on the composition of the milk fats.

Werner (45) examined two samples of skunk fat (*Conepatus suffocans*) from an El Salvador skunk farm with the following results: m.p. 32° and 28° ; n^{40}_D , 1.4612 and 1.4626; $d_{40/40}$, 0.9020 and 0.9010; acid value, 0.75 and 0.75; iodine number (Hanus), 77.5 and 81.0; saponification value, 193.7 and 194.4; Reichert-Meissl value, 1.9 and 2.7; Polenske number, 3.0 and 4.6; and unsaponifiable matter, 0.40 and 0.44 per cent.

Hilditch & Paul (46) have reported their examination of the abdominal fat of a very young Ceylon lizard, known locally as kabara-goya (*Varanus Salvator Laur.*). It is a large carnivorous lizard found in marshy localities in many parts of southeastern Asia. The golden-yellow fat, which is almost completely liquid at 20° , gave the following characteristics: saponification equivalent 283.9, iodine value 70.8, unsaponifiable matter 1.6 per cent, and acid value 4.5. The percentages of component fatty acids of the fat were as follows: myristic 4.2, palmitic 29.3, stearic 9.8, C_{16} unsaturated 12.3, C_{18} unsaturated 39.6, and C_{12} unsaturated 4.8. This data, as well as that of previous investigators, indicated that the lizard fatty acids are intermediate between those of land and aquatic animals. Hepburn & Miraglia (47) found that turkey-egg yolk contained 14.98 per cent of fat having the following characteristics: acid value 7.7, iodine number (Hübl) 85.8, saponification value 184.7, and n^{40}_D 1.4638.

FATS OF AQUATIC FAUNA AND FLORA

The unsaponifiable matter in sperm-whale blubber oil has been investigated by Ueno & Koyama (48). They found that it contained octyl, decyl, and lauryl alcohols, the unsaturated alcohols known as agrophyl ($C_8H_{16}O$), macrocephalyl ($C_{10}H_{20}O$), and adontocetyl ($C_{11}H_{22}O$), and a mixed ether which appears to be $C_{10}H_{21}O$ C_8H_{17} . Minor constituents of marine animal oils have continued to receive attention on the part of Japanese investigators. Toyama & Tsuchiya (49) isolated from sperm-whale oil $\Delta^{5:6}$ -tetradecenyl (physeteryl) alcohol ($C_{14}H_{27}OH$) and $\Delta^{9:10}$ -hexadecenyl (zoomaryl) alcohol ($C_{16}H_{31}OH$). From sperm-blubber oil Toyama & Akiyama (50) obtained very small quantities of $\Delta^{9:10}$ -hexadecenyl, eicosatetraenyl (catadonyl) ($C_{20}H_{35}OH$), and docosapentaenyl (clupanodonyl) ($C_{22}H_{35}OH$) alcohols. Toyama & Tsuchiya (51) isolated and determined the structure of $\Delta^{9:10}$ -decenoic and $\Delta^{5:6}$ -dodecenoic acid from sperm-whale head oil. In sperm-blubber oil they found $\Delta^{5:6}$ -tetradecenoic and $\Delta^{5:6}$ -dodecenoic acids. Ueno & Koyama (52) also examined sperm-whale blubber oil and reported traces of octyl, decyl, and dodecyl alcohols, in addition to the unsaturated alcohols, $C_{10}H_{19}OH$, $C_{11}H_{21}OH$, and $C_{18}H_{35}OH$. Ueno & Yonese (53) reported that small quantities of a hexacosapentaenoic acid ($C_{26}H_{42}O_2$) named "shibic" and another called thynnlic acid ($C_{26}H_{40}O_2$, a hexacosahexaenoic acid) occur in tunny-liver oil.

Brocklesby (54) has reported that Canadian pilchard oil contains approximately the following percentages of acids: myristic 5.1, palmitic 14.4, stearic 3.2, C_{14} unsaturated 0.1, C_{16} unsaturated 11.8, C_{18} unsaturated 17.7, C_{20} unsaturated 17.9, C_{22} unsaturated 13.8, and C_{24} unsaturated 15.2. Lund (55) has summarized the results on record made during a period of 25 years, covering the examinations of whale oil from different species and different localities, including analyses of the oil from different parts of the animal. Tveraen (56) has published a similar review, giving the results of the examination of many samples of oil from the blue whale, including in some cases detailed component acid analyses. Green & Hilditch (57) have made an investigation of the polyethenoid acids of the *n*-octadecane (C_{18}) series present in cod-liver, whale, and carp oils. The general procedure was to prepare by repeated fractional distillation a specimen of the methyl esters of the unsaturated C_{18} acids of each of the three oils. The acids obtained from these esters were separated into three

groups by means of their lithium salts as follows: group 1, acids having lithium salts soluble in acetone; group 2, lithium salts insoluble in acetone but soluble in 80 per cent alcohol; and group 3, lithium salts insoluble in acetone and alcohol. The acids of each group were brominated and the resulting products were separated in each case into three groups: those insoluble in ether, those soluble in ether but insoluble in petroleum ether, and those soluble in both solvents. From the examination of these bromine-addition products, it was found that about 90 per cent of the acids from whale oil were oleic and its isomers and about 3 per cent were tetraethenoid (stearidonic), besides small quantities of dienic acids. In the case of cod-liver oil, about 70 per cent of the acids were oleic and its isomers, about 10 per cent were tetraethenoid, and the remaining 20 per cent contained no linolenic and little, if any, linoleic acid. Of the total C_{18} acids from the liver oil of the tropical carp (*Clenopharyngodon idellus*), tetra- and hexabromostearic acids were obtained, which were equivalent to 6 per cent of linoleic acid and 11 per cent of linolenic acid. The oil from the Guinea and elephant grasses upon which this carp feeds were found to contain notable quantities of linoleic and linolenic acids. Heretofore, it was believed that the oil of whale and various salt-water fishes contained linoleic acid, but this investigation shows definitely that they do not contain detectable quantities.

The liver oil from the large spotted dogfish, *Scyllium stellare*, was examined by Marcelet (58). The livers contained 44 per cent of oil which had 0.90 per cent of unsaponifiable matter, and gave an iodine number (Hanus) of 178, a saponification value of 185, an acetyl number of 47.6, and a refractive index at 29° of 1.4815. The fatty acids separated from the oil contained 32 per cent of saturated constituents in which dorosomic acid ($C_{17}H_{34}O_2$) was identified. Other characteristics of the fat and those of the fatty acids are given. Tsujimoto (59) examined the liver oil from the man-eating shark, *Carcharodon carcharias*, which gave the following characteristics: $d_{15/4}^{20}$ 0.9199, n_D^{20} 1.4733, saponification value 178.1, iodine number (Wijs) 105.9, and unsaponifiable matter 6.97 per cent. The pale yellow insoluble fatty acids which melted at $33-34^\circ$ gave upon bromination 18.7 per cent of ether-insoluble bromides. The unsaponifiable fraction contained 50.5 per cent of cholesterol. The sterol-free portion was found to consist chiefly of selachyl and batyl alcohols.

During the past year, Lovern (60) has continued his extensive study on marine animal fats. The present contribution deals with

specific peculiarities in depot-fat composition of fresh- and salt-water fishes, together with suggestions as to how some of these modifications have been brought about. Data are given on the composition of the fatty acid mixtures of the liver fats of pollock, turbot, salmon, catfish, angel fish, ratfish, and spotted dogfish, as well as that of the body fats of turbot, halibut, sea trout, brown trout, and lampern. On the basis that type differences in fat composition are concerned chiefly with percentages of unsaturated acids, graphs have been prepared using these data by which it is also possible to get a conception of the average type of the normal food fat ingested by most species of fish. The degrees of average unsaturation of one or more of the acid groups, as pointed out, may be characteristically abnormal in certain fish. For example, in the fats of elasmobranch fish certain acids may be either unusually saturated or unsaturated. Both of these types of peculiarity may be due to an appropriate modification of a reversible hydrogenation-dehydrogenation enzyme system. In many cases, the acids of the relatively saturated type are accompanied by chimyl, batyl, and selachyl alcohols, and these may have been formed by different modifications of the hydrogenation system. In fats from some other fish, the increase or decrease in the content of a certain acid or acids is apparently without reference to the other acids present. Examples are the high content of C_{18} acids in fats of rat- and catfishes, and little or no C_{14} acids in that from the tunny. Attention is also called to cases in which two opposing sets of specific requirements are found in the same fish and, where this situation occurs, a compromise results. The examples given include the brown trout in which the characteristics of the salmon family clash with the effects of the fresh-water habitat of the fish. The case of the lampern affords a good illustration of the partially neutralizing effect on the composition of the fat by its feeding both on fresh- and salt-water foods, the composition of lampern fat falling between that of typical fresh-water and marine fishes.

PHOSPHATIDES

In a discussion of the chemistry and industrial utilization of phosphatides, Working (61) states that indirect evidence indicates that a large proportion of the lecithin in animal and vegetable tissues is in combination with proteins. It appears to the reviewer that there is as good, or possibly somewhat better, evidence that phosphatides occur quite generally in combination with carbohydrates. Rewald (62)

also discusses the production, properties, and uses of phosphatides. Tait & King (63) give results obtained on the oxidation of lecithin and other fatty substances in the presence of glutathione. Under the title "Physical Chemistry of the Lipoids," Spiegel-Adolf (64) discusses the action of acids and bases on cephalin from the human brain. Various methods for the determination of phosphatides in blood serum are reviewed and compared by Man (65).

According to Rewald (66), nearly 63 per cent of the phosphatides present in wheat-germ oil are in combination with carbohydrate, protein, or other substances. The total quantity of phosphatides reported in the oil was 0.61 per cent of which eight-tenths was lecithin, the remainder being cephalin. Wieschahn (67) has reviewed soy-bean phosphatides and their uses, giving 64 references.

An examination of the phosphatides extracted from soy beans by hot ethyl alcohol has been made by McKinney, Jamieson & Holton (68). Soluble and insoluble fractions of the extracted phosphatides, obtained by the use of ether, acetone, and alcohol, were found to contain the following constituents: (a) non-protein nitrogenous compounds containing no phosphorus (which are not identified); (b) monoamino-diphosphorus compounds soluble in acetone; (c) diamino-monophosphorus compounds (acetone- and ether-soluble); (d) monoamino-monophosphorus compounds which contained only half as much nitrogen and phosphorus as cephalin and lecithin. These substances, which are insoluble in acetone, were fractionated into (a) a compound insoluble in cold ethyl ether about fifty per cent of which was probably cephalin, (b) a lecithin- β -glycoside complex (soluble in cold alcohol). The glycoside appears to be a combination of a monosaccharide with a dibasic dihydroxy acid (m.p. 103°) having a molecular weight of about 615.

FATTY ACIDS

Bosworth & Helz (69) have isolated an optically active monohydroxypalmitic acid ($C_{16}H_{32}O_3$) from butter fat. The lead soap was soluble in ether and the barium soap in benzene. Different samples of the acid melted from 16.5 to 17.5°. The specific rotation was about +2.45°. In no case was it possible to obtain the acid entirely free from traces of other acids. It is believed that the acid has not been previously described.

Farmer & Van den Heuvel (70) have reinvestigated puniic acid

from the seed oil of the pomegranate, *Punica granatum*, previously described by Toyama & Tsuchiya (71) as a new isomeric elaeostearic acid; it appeared desirable to obtain additional evidence regarding its identity in view of the economic importance of elaeostearic glycerides as well as the biological interest in the relative distribution of conjugated and non-conjugated types of polyene acids. Although the evidence which had been presented indicated that the new acid consisted entirely or largely of a member of the elaeostearic group, the chief points of doubt concerned the homogeneity of the acid isolated and its isomeric relationship to the well-known α - and β -elaostearic acids. The present investigation showed that the X-ray spacings of the acid were different from those given by the other two acids or a mixture of them, and that it failed to give a maleic anhydride derivative. The results of hydrogenation, of alkaline permanganate oxidation, and of its conversion by ultraviolet light into the β -acid definitely proved that the substance was in fact a new elaeostearic acid.

A further investigation has been made by Green & Hilditch (72) on the oxidation by aqueous alkaline permanganate solutions of various polyhydroxy and polyethylene higher fatty acids. As previously observed by Lapworth & Mottram (73), 9,10-dihydroxystearic acid (m.p. 131°) was found to give upon oxidation suberic, octoic, and oxalic acids, there being no scission of the carbon chain between the ninth and tenth carbon atoms. The present investigators have found that this type of oxidation occurs under similar conditions with the 9,10-dihydroxystearic acid (m.p. 95°), 9,10-dihydroxypalmitic acids (m.p. $123-4^\circ$ and 83°), 13,14-dihydroxybehenic acids (m.p. 128° and 100°), and 6,7-dihydroxystearic acid (m.p. 122°). They found that the course of the oxidation was independent of the temperature, the stereoisomeric form of the acid, the length of the carbon chain, or the position of the adjacent hydroxyl groups in the chain. However, it was observed that resistance to oxidation increased in proportion to the increase in distance between the carboxyl and the hydroxyl groups. Under similar conditions, tetrahydroxystearic acids (m.p. 155° and 173°) and the hexahydroxystearic acids (m.p. 169° and 203°) were oxidized, as well as α - and β -elaostearic acids. The dibasic acids produced consisted mainly of azelaic acid and about 20 per cent of suberic acid.

Birose (74), who previously found that the bromination of the fatty acids from lumbang oil gave only one solid linoleic acid tetrabromide (m.p. 114°), has supplemented his investigation with a study

of the linoleic acid from cottonseed and soybean oils. He reported that but one solid tetrabromide (m.p. 114°) is obtainable from the fatty acids of these two oils or by the bromination of the linoleic acid regenerated from the tetrabromide; this is in agreement with his earlier observations. Brown & Shinwara (75) described a method for preparing pure oleic acid from olive oil based on first separating the saturated acids from the mixed fatty acids by crystallization from an acetone solution cooled to -20° and then crystallizing the unsaturated acids by cooling to -60° . After three recrystallizations of the unsaturated acid from acetone at this temperature, a partial crystallization is made at -35° to remove any palmitic acid not separated by the original crystallization at -20° . Brown & Stoner (76) prepared linoleic acid, of purity up to 93 per cent, from the fatty acids of cottonseed and corn oils, and prepared their methyl esters by crystallization at temperatures of -20° , -40° , and -80° .

Cole & Cardoso (77) of the International Leprosy Center, Rio de Janeiro, Brazil, have prepared pure hydnocarpic and chaulmoogric acids as well as their ethyl esters. In each case, the characteristics were determined as well as melting-point curves for mixtures of hydnocarpic and palmitic acids and hydnocarpic and chaulmoogric acids. As stated by the authors, the published data on the physical characteristics of these acids and esters are incomplete and in many cases inaccurate. Komori & Ueno (78) reported that they isolated a new unsaturated acid, $C_{10}H_{18}O_2$, from the oil of *Rindera obtusiloba* which, upon hydrogenation, gave capric acid. The authors suggest that it be known as obtusilic acid. Evidently, it occurs as a minor constituent because only about three grams were separated from five kilograms of the oil.

The study and application of the Diels-Alder maleic anhydride reaction (79) with conjugated ethenoid systems is receiving much attention. When applied to the determination of conjugated double bonds in the acid constituents of various oils it is commonly known as the "diene value"; this, in terms of iodine, is calculated to be in conformity with other measurements of unsaturated linkages. Morrell & Davis (80) have investigated the reaction of maleic anhydride with the esters of α - and β -elaeostearic acid paying special attention to the constitution of the products formed. They found that maleic anhydride unites with the $\Delta^{11:12}, \Delta^{13:14}$ -ethenoid linkage of the α -acid, whereas it combines with the $\Delta^{9:10}, \Delta^{11:12}$ -ethenoid group of the β -acid. The α -acid addition product upon exposure to oxygen gives a keto-

alcohol which is formed by oxidation taking place at the $\Delta^{9:10}$ double bond, but the β -acid addition compound is oxidized at both the ethenoid linkage and the double one (Δ^{18}) which is remote from the carboxyl group. With hydrogen peroxide, however, it was found that in each case the oxidation took place only at the remaining chain double linking to form dihydroxy derivatives.

Moore (81) has further investigated the effect of prolonged treatment with potassium hydroxide on the absorption spectra of fatty acids from various vegetable and animal fats. From the experiments made it was concluded that saturated and oleic acids, even after heating for twenty-four hours with potassium hydroxide, do not show the property of increased spectroscopic absorption as do the polyethylenic acids. Under the experimental conditions used, the fatty acids of vegetable oils, excepting those from tung oil, showed increased absorption with the degree of unsaturation. The absorption maxima were at about 230 μ . The acids from tung oil were exceptional in that the oil, even before saponification, showed a maximum absorption (about 270 μ). The acids of land mammals' fats differed from those of vegetable origin in having a considerable portion, and sometimes practically all, of their potential absorption developed in the natural state. The absorption maxima were again at about 230 μ . The acids of marine oils differed from those of vegetable and land animals in developing absorption (upon prolonged alkali treatment) at about 270 μ together with that at 230 μ , with but slightly lower intensity. A detailed study of the effect of refluxing the mixed fatty acids of linseed oil with alcoholic potassium hydroxide solution was made to determine the time required for the changes to proceed to completion. The first effect observed was to raise the absorption at 230 μ . Later, absorption at 270 μ began to develop, and after about a week's treatment its intensity of absorption almost equaled that at 230 μ . This latter development coincided with the formation of a solid unsaturated acid. This acid was separated in crystalline form and bore a striking resemblance to the elaeostearic acids prepared from tung oil but it had a higher melting point (77°) than the α - and β -isomerides. After separation of the solid acid, the mother liquors upon further treatment with alkali failed to produce any more of the solid acid and showed an absorption only at 230 μ . This solid unsaturated acid was evidently derived from the linolenic acid, as a similar experiment made with corn-oil fatty acids which contain no linolenic acid gave none of this solid unsaturated acid.

Mention also should be made that the solid acid upon bromination gave no ether-insoluble hexabromide, which is also the case with the elaeostearic acids.

CONSTITUENTS OF WAXES

Greene & Foster (82) were the first investigators to show that the oil which constitutes about 50 per cent of the seeds of *Simmondsia californica* is a liquid wax. Green, Hilditch & Stainsby (83) found that the chief acid in this wax was $\Delta^{11:12}$ -eicosenoic, accompanied by smaller quantities of docosenoic acid. The principal alcohols with which the acids were combined were shown to be $\Delta^{13:14}$ -docosenol and $\Delta^{11:12}$ -eicosenol. McKinney & Jamieson (84), who also made an investigation of this wax, found that it had the following percentages of fatty acids and alcohols: saturated acids 1.64, palmitoleic acid 0.24, eicosenoic acid 30.30, docosenoic acid 14.20, eicosenol 14.60, and docosenol 33.70. Tests for glycerine were negative. It should be mentioned that at the present time no other seed is known which fabricates a wax in place of a fat.

Kuwata & Ishii (85) examined wool grease and reported that it contained lanomyristic acid ($C_{14}H_{28}O_2$), lanopalmitic acid ($C_{16}H_{32}O_2$), and traces of lanostearic and lanoarachidic acids. Besides these acids evidence was obtained that there are a considerable number of liquid saturated acids. They state that the acids in wool grease are not identical with the normal fatty acids. Abraham & Hilditch (86), who also investigated this product, failed to detect any normal palmitic, stearic, or cerotic acids and concluded that the chief acid components are probably members of a cyclic series. They suggested that the acids in wool grease may be structurally related to or derived from the sterols, with which they are combined as esters, and that they may be derived from an isoprene or terpene foundation rather than an unbranched chain of carbon atoms.

Markley & Sando (87) have reported the results of their investigation on the petroleum- and ethyl-ether soluble constituents of the skin of the Bing cherry (*Prunus avium*, L.). The moisture-free skins were first extracted with petroleum ether, then with ethyl ether. The first extract amounted to 0.8 per cent and the second to 0.1 per cent of the skins. The petroleum-ether extract consisted of a mixture of oleic, linoleic, palmitic, and stearic acids and a small quantity of an acid of a higher molecular weight, a small quantity of glycerol, and a hydrocarbon fraction in which nonacosane ($C_{29}H_{60}$) was present.

The ether extract yielded ursolic acid ($C_{30}H_{48}O_8$) and *d*-glucosidyl-sitosterol ($C_{35}H_{60}O_6$) which melted at 265–266°.

Reeves & Anderson (88) have investigated the chloroform-soluble wax which, as shown by Anderson & Roberts (89), amounts to 70 per cent of the total lipids from avian tubercle bacillus or 10.8 per cent of the dried bacilli. The wax was a non-crystalline, light yellow powder which was readily soluble in chloroform, ether, benzene, toluene, ligroin, and ethyl acetate, but insoluble in acetone, ethyl and methyl alcohols. It melted at 53–54° and gave a saponification value of 77, an iodine number of 7.8, and $[\alpha]_D$ of +25.6° in a chloroform solution. The crude wax was purified by repeated precipitation with methyl alcohol from a chloroform solution. After forty such treatments, a fraction was obtained which gave an optical rotation of 38.6°. It melted at 54–55° and gave an iodine number of 4.5. A second purified fraction from the alcoholic mother liquors gave an optical rotation of +17.7°, an iodine number of 8.7, and melted at 53–55°. Except for a lower optical rotation and somewhat greater solubility, fraction 2 was very similar to fraction 1. The chief constituents of the wax are hydroxy fatty acids of very high molecular weight, the disaccharide trehalose, and the saturated alcohols, *d*-cicosanol-2 and *d*-octadecanol-2. Tests for glycerine were negative.

Markley, Nelson & Sherman (90) identified the following constituents in the non-volatile, waxy residue remaining after distillation of the volatile oil pressed from grapefruit peel: linolenic, linoleic, and oleic acids, a sapogenic ketone ($C_{30}H_{52}CO$), hydrocarbons ($C_{29}H_{60}$ and $C_{31}H_{64}$), a phytosterol ($C_{28}H_{47}OH$), and umbelliferone ($C_9H_6O_3$).

ALCOHOLS AND HYDROCARBONS

Marcelet (91) reported that the unsaponifiable fraction of peanut oil contains two hydrocarbons, $C_{15}H_{30}$ and $C_{19}H_{38}$, which have been named "hypogene" and "arachidene," respectively. It was stated that the name "hypogene" was suggested because of the relationship of the hydrocarbon to hypogaic acid, which formerly was believed to be a constituent of peanut oil but was later shown to be non-existent in this oil. From a similar examination of the unsaponifiable fraction of olive oil he isolated the following six hydrocarbons: oleatridecadiene ($C_{13}H_{14}$), oleahexadecadiene ($C_{16}H_{20}$), oleanonadecadiene ($C_{19}H_{30}$), oleatricosatriene ($C_{23}H_{42}$), oleaoctacosatetrene ($C_{28}H_{50}$), and oleatetracosane ($C_{24}H_{50}$). Toyama & Akiyama (92) have ob-

tained from sperm-whale blubber oil very small quantities of $\Delta^9:10$, hexadecenyl, eicosatetraenyl (catadonyl), and docosapentaenyl (clupanodonyl) alcohols. Ueno & Koyama (93) found also traces of octyl, decyl, and dodecyl alcohols besides unsaturated alcohols having the composition $C_{10}H_{20}O$, $C_{11}H_{22}O$, and $C_{18}H_{36}O$.

Marcelet (94) extracted raspberries with ether and, after removal of the solvent, cooled the oil to 15° which caused the separation of six per cent of wax. From the unsaponifiable fraction, which amounted to 22.8 per cent of the wax, he separated by crystallization an alcohol ($C_{19}H_{40}O$) melting at 62.5° . The acetate melted at 58° , the benzoate at 45° , and the phenylurethane derivative at 80° . Apparently, no attempt was made to isolate any other of the wax constituents.

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THE CHEMISTRY OF AMINO ACIDS AND PROTEINS*

By MAX BERGMANN AND CARL NIEMANN

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For a number of years the biological significance of the proteins has been obscured by the isolation of numerous non-protein substances possessing striking physiological properties, and it has been suggested that the proteins act merely as the passive carriers of physiologically active, or prosthetic, groups. Recently, a relatively large number of enzymes, hormones, viruses, and toxins have been obtained as crystalline proteins, and the question has been raised as to whether the biological activity is to be attributed to some unknown prosthetic group or to the intrinsic nature of the protein molecule itself. This situation has stimulated interest in protein chemistry, and in the period covered in this review more than 500 papers have appeared. The reviewers have taken the position that knowledge of the chemical structure of the protein molecule is of primary importance as a basis for the physico-chemical and biological investigation of proteins and therefore have assumed the liberty of emphasizing the chemical and biochemical studies.

AMINO ACIDS AND PEPTIDES

Synthesis.—Two years ago Rose and his coworkers [cf. *Ann. Rev. Biochem.*, **6**, 269 (1937)] isolated an α -amino- β -hydroxy-*n*-butyric acid (threonine) from a fibrin hydrolysate and found that this amino acid was essential for growth. In order to make the new amino acid more accessible, Carter and coworkers (202, 203, 207) have investigated the possibility of its synthetic preparation from crotonic acid and have obtained both *d*- and *l*-threonine in fair yields. Kitagawa & Takani (106, 107) have synthesized canalin and have offered additional evidence which supports the formulation of canavanin as $\text{NH}_2 \cdot \text{C}(\text{NH}) \cdot \text{NH} \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{CO}_2\text{H}$. Wilson and his associates (97, 140) and Akasi (5), with the aid of synthetic experiments, have concluded that octopine (pectenine) is *l*- α -propionic acid-*l*(+)-arginine. Robson (35) has continued his investigations on the synthetic preparation of α -amino acids and has

* Received January 3, 1938.

shown that *dl*-tryptophane can be prepared in good yield and purity by condensing indole-3-aldehyde with hydantoin in the presence of piperidine, followed by the reduction and hydrolysis of the condensation product with ammonium sulfide. This investigator has also described the preparation of carbamido-arginine (33) and its conversion into ornithine, ornithuric acid, and benzoylornithine (34). Schiltz & Carter (168) have synthesized *dl*-serine from methyl acrylate in 30 to 40 per cent yields with the aid of the following reactions:

$$\begin{aligned} \text{CH}_2 = \text{CH} \cdot \text{CO}_2\text{CH}_3 &\rightarrow \text{CH}_2(\text{OCH}_3) \cdot \text{CH}(\text{HgOAc}) \cdot \text{CO}_2\text{CH}_3 \rightarrow \\ &\text{CH}_2(\text{OCH}_3) \cdot \text{CH}(\text{HgBr}) \cdot \text{CO}_2\text{CH}_3 \rightarrow \text{CH}_2(\text{OCH}_3) \cdot \text{CHBr} \cdot \\ &\text{CO}_2\text{CH}_3 \rightarrow \text{CH}_2(\text{OCH}_3) \cdot \text{CHBr} \cdot \text{CO}_2\text{H} \rightarrow \text{CH}_2(\text{OCH}_3) \cdot \\ &\text{CH}(\text{NH}_2) \cdot \text{CO}_2\text{H} \rightarrow \text{CH}_2(\text{OH}) \cdot \text{CH}(\text{NH}_2) \cdot \text{CO}_2\text{H}. \end{aligned}$$

Signaigo & Adkins (172), starting with pyrrole, have prepared *dl*-proline in 55 to 60 per cent yields. Dideuterioleucine was obtained from isovaleraldehyde by Kinney & Adams (105) in the following steps: $(\text{CH}_3)_2 > \text{CH} \cdot \text{CH}_2 \cdot \text{CHO} \rightarrow (\text{CH}_3)_2 > \text{CH} \cdot \text{CHBr} \cdot \text{CH}(\text{OR})_2 \rightarrow (\text{CH}_3)_2 > \text{C} \cdot \text{CH} \cdot \text{CH}(\text{OR})_2 \rightarrow (\text{CH}_3)_2 > \text{CD} \cdot \text{CHD} \cdot \text{CH}(\text{OR})_2 \rightarrow (\text{CH}_3)_2 > \text{CD} \cdot \text{CHD} \cdot \text{CHO} \rightarrow (\text{CH}_3)_2 > \text{CD} \cdot \text{CHD} \cdot \text{CH}(\text{NH}_2) \cdot \text{CO}_2\text{H}$. Dideuteriovaline was prepared from isobutyraldehyde in a similar manner. Deulofeu has applied the azlactone method to the synthesis of 2,4-dihydroxyphenylalanine (52) and has prepared *N*-methyl-3,4-dihydroxyphenylalanine by the condensation of vanillin with creatinine and subsequent hydrogenation and hydrolysis of the condensation product (77). It is of interest to note that urea is a possible by-product of this reaction. Fischer & Feldmann (65) have applied the Strecker synthesis to monoacetone glyceric aldehyde and monoacetone tartaric dialdehyde, and have obtained α -amino- β,γ -dihydroxy-*n*-butyric acid and α,α' -diamino- β,β' -dihydroxy-*n*-adipic acid. An interesting but somewhat questionable experiment is that of Ludwig & v. Mutzenbecher (121) who claim to have isolated thyroxine from an iodinated "unspecific" protein.

Harington and his coworkers (45) have described a novel method for the introduction of carbohydrate into the protein molecule. Acetobromoglucose was condensed with carbobenzoxytyrosine ethyl ester and the resulting *O*- β -tetraacetylglucosido-*N*-carbobenzoxytyrosine ethyl ester was converted into *O*- β -glucosido-*N*-carbobenzoxytyrosylazide via the hydrazide. The azide was coupled with gelatin and the carbobenzoxy residues were removed from this substance by reduction with sodium in liquid ammonia. The resulting glucosidotyrosyl-gelatin contained 4.6 per cent of glucose, and evidence was presented

to show that the glucosidotyrosyl residues were attached to the α -amino groups present in the original gelatin.

Du Vigneaud & Hunt (196) described the synthesis of *d*-carnosine and found that this enantiomorph produced no depressor action when it was applied in amounts that were twenty times greater than those normally used in the case of the active natural or *l*-compound. Du Vigneaud & Behrens (198) have devised a method for protecting the imidazole ring of histidine during certain reactions and have applied this technique to the preparation of *l*-amino-*N*-methylhistidine. The principle of the method depends upon the fact that histidine reacts with benzyl chloride in the presence of sodium and liquid ammonia to form a monobenzyl derivative in which the benzyl residue is attached to the imidazole nitrogen and that this benzyl residue can be removed by reduction with sodium in liquid ammonia. It was also noted that the *p*-toluenesulfonyl group could be removed by reduction in the same solvent; when the *p*-toluenesulfo derivative of benzylhistidine was methylated with methyl iodide the resulting product was converted into *l*-amino-*N*-methylhistidine in one step by reduction with sodium in liquid ammonia. The partial synthesis of anserine has been reported by the same authors (17) to have been accomplished through the coupling of carbobenzoxy- β -alanyl azide with the methyl ester of *l*-1-methylhistidine which in turn had been obtained from natural anserine. Du Vigneaud and coworkers have also accomplished the synthesis of α -glutamylcysteinylglycine (isogluthathione) (197).

Greenstein (75, 76) has continued his studies on the preparation of multivalent amino acids and peptides and has synthesized anhydrocysteinyl-*l*-cysteine. This substance, on oxidation with hydrogen peroxide, was converted into the crystalline dimeric molecule, bisanhydro-*l*-cystinyl-*l*-cystine.

v. Braun, Dengel & Jacob (36) have prepared a large number of *N*-substitution products of decarboxypeptides (diglycyl-decarboxyllysine, dialanyl-decarboxyornithine, etc.) in the course of a study on compounds of pharmacological interest.

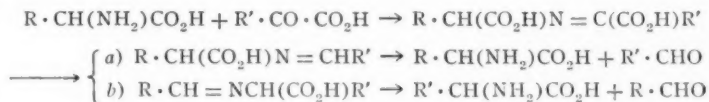
Bergmann & Ross (18) prepared α -hippuryllysineamide by ammonolysis of α -hippuryl- ϵ -carbobenzoxylysine methyl ester and hydrogenation of the resulting amide. It was noted that this α -hippuryllysineamide was split by tryptic proteinase (Waldschmidt-Leitz), forming hippuric acid, lysine, and ammonia. Later Bergmann & Fruton (19) investigated the action of crystalline Northrop trypsin and chymotrypsin on this compound and as these crystalline enzymes

did not split the substrate it was concluded that the splitting observed by Bergmann & Ross was due to a hitherto unknown third tryptic proteinase which was designated "heterotrypsin."

Bergmann & Fruton (19) condensed N-carbobenzoxy-O-acetyl-L-tyrosyl chloride with glycine ethyl ester, and the resulting N-carbobenzoxy-O-acetyl-L-tyrosylglycine ethyl ester was decarbobenzoxylated and coupled with carbobenzoxyglycyl chloride. The syrupy reaction product was converted into the amide by treatment with ammonia. When this carbobenzoxyglycyl-L-tyrosylglycineamide was treated with four times recrystallized Northrop chymotrypsin a rapid splitting of one peptide bond was noted and one of the split-products was identified as carbobenzoxyglycyl-L-tyrosine. A number of other peptides were investigated and it was observed that N-carbobenzoxy-L-tyrosylglycineamide and glycyl-L-tyrosylglycineamide were also readily split by chymotrypsin. On the other hand, carbobenzoxyglycyl-L-phenylalanylglycineamide and N-carbobenzoxy-L-tyrosylglycylglycineamide were attacked much more slowly; N-carbobenzoxy-L-tyrosylglycine, carbobenzoxyglycyl-L-glutamylglycineamide, benzoylglycyl-L-lysineamide, carbobenzoxyglycyl-L-leucylglycineamide, benzoyl-L-leucyl-L-leucylglycine, and chloroacetyltyrosine were not split at all by chymotrypsin. Aside from the practical advantage of possessing a substrate of known and reproducible composition for studies of enzyme specificity the knowledge that chymotrypsin, which is generally recognized to be a true proteinase, will split carbobenzoxyglycyl-L-tyrosylglycineamide, glycyl-L-tyrosylglycineamide, and N-carbobenzoxy-L-tyrosylglycineamide is of great importance as it demonstrates that the enzyme is capable of degrading genuine proteins [see also (24)] are equally capable of hydrolyzing the simple peptide bond present in the above compounds (see the section on the structure of proteins). In a similar study Bergmann, Fruton & Pollok (24) found that benzoylarginineamide was readily hydrolyzed by Northrop crystalline trypsin but was not hydrolyzed by chymotrypsin; thus the authors were able to demonstrate a differentiation of the pancreatic trypsins on the basis of the enzymic specificities. Bergmann, Fruton & Fraenkel-Conrat (21) have been able to show that liver cathepsin, papain, and bromelin exhibit different specificities towards synthetic substrates.

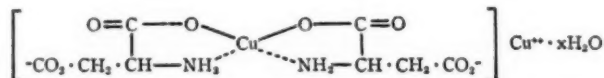
Reactions.—In view of the utility of the azlactone method for synthesizing amino acids and peptides, the knowledge that diazomethane in the presence of methanol will split azlactones to form the methyl ester of the N-benzoyl-dehydroamino acid (64) is particularly inter-

esting. The use of diazomethane for the methylation of amino acids has been shown by Kuhn & Brydowna to lead to the formation of methyl ester and betaine in various amounts, depending on the nature of the amino acid; these results have been interpreted on the basis of the *zwitterion* hypothesis (110). The reaction between α -ketonic and α -amino acids has been studied by Herbst (84), and the following reaction mechanism has been proposed:



The deamination of histidine by ascorbic acid has been investigated by Holtz & Triem (90, 91) and by Edlbacher & v. Segesser (57); it appears that the reaction is not an asymmetric one as was claimed in the preliminary communication (90). The oxidation of amino acids by hypochlorite or hypobromite has been the subject of several investigations (70, 148, 209) and it has been found that with hypobromite the extent of aldehyde or nitrile formation depends upon the alkalinity of the reaction mixture. The greater the alkalinity, the greater is the tendency to produce aldehyde at the expense of nitrile. However, nitrile formation is increased with an increasing chain length of the original amino acid (70). Through the electrolytic oxidation of α -amino acids, acids of one less carbon atom are obtained; it was suggested that this reaction proceeds through an aldehyde stage (188). Similarly, it has been shown that persulfate in an alkaline solution will convert an amino acid into ammonia and an aldehyde of one less carbon atom, but the maximum amount of aldehyde produced is not over 45 per cent (113; see also 112). Kanewskaja (100) has studied the Hofmann degradation of acylated amino acid amides and in the case of benzoylglycineamide obtained, as might be expected, benzoylmethylene diamine hydrochloride. However, the degradation of benzoyl- β -alanine led to the formation of benzoic acid and glyoxalidone-(2) [ethylene carbamide]. The reaction of amino acids with formaldehyde has continued to be a topic of investigation (118, 160, 200); Ratner & Clarke (160) have found that formaldehyde reacts with cysteine over a wide pH range to form thiazolidine-4-carboxylic acid. Woodward & Schroeder (208) studied a similar reaction and found that cysteine will react with acetone to form 2,2-dimethylthiazolidine-4-carboxylic acid; it was noted, however, that this compound is un-

stable in aqueous solutions. Pfeiffer & Werner investigated the composition of copper aspartate and copper glutamate and proposed the following formula for the aspartic acid compound (154):



A similar formula was advanced for copper glutamate, and it was established that the Cu^{++} is replaceable by sodium, barium, etc. Virtanen & Laine (199) have made the interesting observation that living legume bacteria are capable of decarboxylating *L*-aspartic acid, thereby forming β -alanine in practically quantitative yields. Arnow (10) has claimed that dihydroxyphenylalanine is formed on exposure of tyrosine solutions to ultraviolet radiation.

Optical configuration.—The spatial configuration of natural α -amino- β -hydroxy-*n*-butyric acid (threonine) has been shown to be analogous to that of *d*(−)-threose and the natural amino acid has been designated *d*(−)-threonine (129). It should be pointed out that although natural threonine is designated *d*(−), it is also configurationally related to *L*-alanine and is therefore a member of the *L*-series of amino acids. The view that all natural amino acids are members of the *L*-series has received additional confirmation through the investigations of Pfeiffer & Christeleit (153), Levene & Mardashev (116, 117), and Schneider (169). On the basis of the configurational relationship existing between natural norleucine and 2-aminoheptane (117), Rainey (159) has suggested the use of Boys' scheme of representation for determining the absolute configuration of the natural α -amino acids. Reihlen & Knöpfle (161) have shown that dextro-rotatory α -aminophenylacetic acid is the *l*(+) form.

Bruckner & Ivánovics (37) have isolated from *Bacillus mesentericus vulgatus* considerable quantities of laevorotatory glutamic acid which is the unnatural or *d*(−)-glutamic acid. The original observation of Jacobs & Craig [*Ann. Rev. Biochem.*, **6**, 529 (1937)] as to the presence of unnatural or *d*-proline in the various ergot alkaloids has been confirmed by Smith & Timmis (173). In view of the above isolation of *d*-glutamic acid it may be of interest to recall that in many schemes of phytochemical genesis glutamic acid is considered to be the precursor of proline. Conrad & Berg (48) maintained rats on a histidine deficient diet supplemented with *d*-histidine; on examination of the tissues, essentially pure *L*-histidine was obtained. Kotake &

Goto (108), using rat kidney slices, found a similar inversion of *d*-tryptophane into *l*-tryptophane. From these experiments it may be concluded that, in some cases, the unnatural form of an amino acid is susceptible to inversion by the living organism. It should be pointed out that this inversion may proceed through an intermediate containing no asymmetric carbon atom, such as an α -keto acid. In this connection, it may be mentioned that Heinsen (83) claims to have isolated *dl*-arginine from a kidney autolysate.

Determination.—The Van Slyke method for the gasometric determination of amino nitrogen has proved to be of inestimable value in the chemistry of amino acids and proteins. It is therefore of interest to note that Van Slyke & Dillon (194) have now described a gasometric method for the determination of the carboxyl groups in α -amino acids. These investigators found that at pH 5, or somewhat lower in aqueous solutions, carbon dioxide from the carboxyl groups adjacent to the α -amino groups was quantitatively split off in 3 minutes by ninhydrin (triketohydrindene hydrate). The Van Slyke manometric apparatus was used to measure the gas evolved. Acetic acid, citric acid, and lactic acid gave no carbon dioxide under the above experimental conditions. With the exception of the dicarboxylic acids, all of the naturally occurring α -amino acids, including proline, yielded 1 mol of carbon dioxide. Aspartic acid gave 2 mols of carbon dioxide immediately, and glutamic acid 2 mols more slowly. It is noteworthy that peptides did not yield carbon dioxide when treated with ninhydrin under the above conditions. As the authors point out, this new method in conjunction with the gasometric amino nitrogen method should be very useful for distinguishing between amino acids and peptides in complex mixtures. The application of these methods to the problems of enzyme chemistry is obvious, and it is to be anticipated that many of the present difficulties will find ready solution through their use.

Kendrick & Hanke (104) have proposed a modification of the Van Slyke manometric amino nitrogen method, and by the use of potassium iodide in the reaction mixture have obtained theoretical values for glycine and cystine. A manometric procedure for the estimation of arginine, with arginase, has been described by Hunter & Pettigrew (96). As the result of many determinations, these authors conclude that 15.7 per cent of the total nitrogen of gelatin is arginine nitrogen. (This value corresponds to 8.8 gm. of arginine per 100 gm. of gelatin with a nitrogen content of 18.0 per cent.) Town (192) has

found that nitranilic acid (2,5-dihydroxy-3,6-dinitro-*p*-benzoquinone) forms a diglycine salt the solubility of which in water is 0.8 per cent and which is practically insoluble in 80 to 100 per cent ethanol. With this reagent Town isolated sufficient diglycine nitranilate from a gelatin hydrolysate to account for 25.9 gm. of glycine per 100 gm. of protein. Caseinogen gave 3.4 per cent of glycine.

The procedure advocated by Chibnall & Westall for the determination of glutamine in plant tissues has been revised in the light of more recent information reported by Vickery, Chibnall, and co-workers (195). As the amide group was found to be completely hydrolyzed when glutamine was heated at 100° for 2 hours at pH 6.5, the ammonia liberated under these conditions is a measure of the glutamine content. Asparagine does not interfere. The estimation of the amide nitrogen present in ovalbumin has been studied by Shore and coworkers (171); it was concluded that this protein contains 24 equivalents of amide nitrogen per mol.

Gurin (78) has found that N-benzenesulfonyl amino acid esters, N-benzenesulfonyl di- and tripeptide esters, and N-carbethoxy peptide esters can be distilled at pressures of 10^{-6} and 10^{-7} mm. without racemization and with very little decomposition. An artificial mixture containing N-acyl amino acid, dipeptide, and tripeptide was fractionally distilled and the individual components were isolated in a pure form and in yields of 90 per cent or more. In a subsequent paper Gurin (79) prepared crystalline helianthates of various amino acid and polypeptide esters by adding methyl orange to the ester hydrochlorides. These salts were non-hygroscopic and were sufficiently insoluble in water to serve as a means of isolation and characterization. A new method for the separation of amino acids, which is based upon the differential solubility of the various amino acids in fatty acids (i.e., acetic, propionic, and butyric acids), has been proposed by v. Przylecki & Kasprzyk (157). It is to be regretted, however, that the authors did not submit their procedure to a crucial test, i.e., the actual analysis of a protein.

Holiday (88) has described a spectrophotometric method for the estimation of tyrosine and tryptophane in proteins; in those cases where the protein under investigation is soluble in water or in weak acid or alkali this method appears to be quite useful. The colorimetric determination of tyrosine and tryptophane has been considered by Lugg (122) who has devised a new method for the estimation of these amino acids. It should be pointed out that the investigation was con-

cerned only with the estimation of tryptophane and tyrosine existing as such in solution, and consequently it is doubtful that these methods will find immediate application in the analysis of proteins. A number of other colorimetric methods have been described during the past two years; without considering their advantages or disadvantages, attention is called to the methods devised for the estimation of the components of 3,4-dihydroxyphenylalanine mixtures (9), the determination of histidine in the presence of carnosine (128), and the estimation of glucosamine in proteins (146).

Abderhalden & Neumann (1) reported that polypeptides containing glycine in the initial position (free glycine amino group) gave a positive reaction with the *o*-phthaldialdehyde reagent of Zimmermann, whereas glycine in a central or terminal position (free glycine carboxyl group) gave no reaction. Accordingly, this method was suggested for the allocation of glycine in peptides of unknown structure.

The reactions of amino acids with alkaloidal reagents and salts of heavy metals to give distinctive types of crystals has been suggested (185) as a means for the microscopic identification of amino acids. The use of copper salts for the same purpose has been suggested by Kirk and coworkers (50). Town (193) has extended his investigations on the separation of amino acids by means of their copper salts and on working over the methanol-soluble copper salt fraction obtained 10.3 per cent proline from gliadin.

Balson, Earwicker & Lawson (14) have studied the potentiometric titration of amino acids and peptides in 90 per cent ethanol and have concluded that the colorimetric titration (indicator) is not reliable in the case of mixtures; hence they advocate the use of the potentiometric method. This finding is of significance in view of the fact that indicator titrations have repeatedly been used in order to investigate the ratio of amino and carboxyl groups liberated during the enzymatic digestion of proteins. Conclusions based on such titrations seem not to have a firm foundation. Dunn & Lashakoff (54) conducted a similar investigation of the formol titration and claim that an accuracy of ± 0.1 per cent is obtained with monoamino-monocarboxylic acids when a glass electrode is used. The titration of amino acids and proteins in glacial acetic acid has been investigated by Harris (80) and by Russell & Cameron (167); from their data the latter authors concluded that gelatin contains 9.6×10^{-4} equivalents of basic amino acids per gm. of protein.

The adsorption of amino acids on Lloyd's reagent and frankonite

was studied by Fuchs (71); it was found that the recoveries on elution were about 80 per cent and varied from 68 to 98 per cent on adsorption, depending upon the nature of the amino acid.

Miscellaneous.—The reviewers wish to call attention to the new edition of Sidgwick's *The Organic Chemistry of Nitrogen* which has appeared during the past year (189). The section on amino acids and proteins is well written and, in addition, the discussions of modern organic structural concepts are of particular interest to those who are engaged in problems involving amino acids and proteins.

Additional papers.—Dissociation constants and the structure of zwitterions (143); the dielectric constants of solutions of dipolar ions (228); Raman spectra of amino acids and related compounds (58, 59); studies in the physical chemistry of amino acids, peptides, and related substances (123); influence of the dielectric constant in biochemical systems (46); the glutamic acid-pyrrolidonecarboxylic acid system (205); the solubilities of *l*-proline, *l*-hydroxyproline, *l*-dihalo-genated tyrosines, and *d*-valine (51, 191, 206); α -amino-*n*-butyric acid as a constituent of proteins (2); the preparation of methionine-free natural leucine (68).

PROTEINS

Structure.—The trend, established in the past decades, towards the use of physical methods in investigations concerned with the structure of proteins has been continued in the last two years in the ultracentrifugal studies of Svedberg and of Wyckoff, in the X-ray investigations of Astbury, Bernal, Wyckoff, and others, in the electrochemical studies of Cohn and of Schmidt, and in the magnetic moment measurements of Pauling. The task of these and the purely chemical studies is the elucidation of the following three special problems: (a) the nature of the essential covalent linkages binding the individual amino acid residues within a given protein molecule; (b) the nature of the subsidiary forces responsible for the individual shape of a given protein molecule; and (c) the total number of amino acid residues and the number and the arrangement of the individual amino acid residues within a given protein molecule.

In a review of recent developments in protein chemistry Grassmann (74) assumed that the proteins have a periodic structure within the peptide chain, such that identical or analogous peptides composed of only a few amino acids are repeated throughout the whole peptide

chain; it was suggested that in egg albumin groups of three amino acids are periodically repeated throughout the molecule. In addition to the isolation of lysylprolylglycine from gelatin [*Ann. Rev. Biochem.*, 6, 186 (1937)] Grassmann regarded the earlier experiments of Waldschmidt-Leitz, on the whole number relationships supposedly found during the stepwise enzymatic degradation of proteins, as suggesting and supporting the above hypothesis. It must be emphasized that the experiments of Waldschmidt-Leitz were conducted with heterogeneous enzymes and substrates; and even if it were possible to demonstrate this supposed whole number relationship on digestion of proteins with enzymes, the reviewers cannot see how such relations could be used either to confirm or to deny the existence of a periodic structure in a given protein molecule.

In another review of the prevailing hypotheses of protein structure Block (32) suggested that the basic amino acid residues present in a given protein molecule constitute an "anlage" and thereby have a directive influence on the structure of the molecule. In this hypothesis the relative ratio of the basic amino acids to each other was held to be of prime importance and, in contrast, the absolute amount of the basic amino acid residues in a particular protein was assumed to be of no significance. The reviewers find two objections to Block's hypothesis. First, if this so-called "anlage" has so decided an effect on the structure of a protein while the latter is being formed, then it is difficult to understand why, for example, wool keratin and silk fibroin (which is classified as a "keratin" by Block), both of which supposedly possess the same "anlage" of basic amino acid residues, have in fact so different a general structure. Second, the variations noted in the experimental ratios are greater than would normally be ascribed to an experimental error; in general, the constancy of ratio that one would expect if the "anlage" theory were correct apparently was not observed.

The structure of proteins has been considered as a problem in topographical geometry by Wrinch (13, 69, 210-217). In general this approach has led to the hypothesis of the so-called "cyclol" structure of proteins, a structure which has several points in common with the earlier "akropeptide" theory of Fodor (67). Wrinch has constructed spatial models of pepsin, insulin, and egg albumin (214-217), which are composed of 288 amino acid residues per molecule of protein and contain as an essential element the so-called "cyclol" structure. The reviewers believe such a structure to be incompatible with

the existing knowledge of enzyme chemistry and classical protein chemistry; in fact, two experiments designed to test the "cyclol" hypothesis failed to support it (99, 103). [The reviewers can form no opinion at the present time as to whether or not experiments with protein surface films either support or deny the existence of the "cyclol" structure since there is no agreement among those familiar with this mode of investigation (26, 42, 114, 131, 138, 139, 144, 145, 155).] Mirsky & Pauling (137), Wrinch & Jordan Lloyd (212, 213), and Huggins (94, 95) have suggested that the linkage known as the "hydrogen bond" or the "hydrogen bridge" may be present in the genuine protein molecule and may be one of the forces responsible for the maintenance of the unique spatial orientation of a given protein molecule. The views advanced by Wrinch & Jordan Lloyd and in part by Huggins are reminiscent of the theories in vogue during the period from about 1920 to 1930. In general these authors look upon the genuine protein molecule as an ensemble of cyclic systems, of low molecular weight, containing from two to six amino acid residues, which have been polymerized by means of the hydrogen bond. It is the opinion of the reviewers that there is little or no experimental evidence which would tend to support the type of structure postulated above and, particularly in the case of the linear molecule, the suggestion of Wrinch & Jordan Lloyd (212) is completely at odds with the known facts of organic and enzyme chemistry. On the other hand, Mirsky & Pauling (137) have employed the concept of the hydrogen bond in an entirely different manner. They suggest that the native protein molecule is a potential linear polypeptide chain which has been cast into a unique spatial orientation and is maintained in this state, at least in part, with the aid of hydrogen bonds.

Bergmann & Niemann (22, 23) have conducted a series of analyses of cattle globin, cattle fibrin, egg albumin, and silk fibroin and from the results of these experiments have concluded that the total number of amino acid residues (N_t), the number of individual amino acid residues (N_i), and the frequency of the individual amino acid residues ($F_i = N_i/N_t$) that are contained in a molecule of protein can be expressed by the following equations: (a) $N_t = 2^n \times 3^m$ where n and m are positive whole numbers; (b) $N_i = 2^{n'} \times 3^{m'}$ where n' and m' are either zero or positive whole numbers; (c) $F_i = 2^{n''} \times 3^{m''}$ where n'' and m'' are either zero or positive whole numbers; (d) $n = n' + n''$; (e) $m = m' + m''$; (f) $N_t = N_i' + N_i'' + N_i''' + \dots + N_i^z$. It was held to be of the utmost significance that

the experimentally determined values of N_1' , N_1'' , N_1''' , etc., and of F_1' , F_1'' , F_1''' , etc., led to values of N_1 which were unique whole number multiples of 288 or $2^5 \times 3^2$. It was emphasized that the various protein molecules fell into groups containing a constant and unique number of amino acid residues rather than into groups of constant weight. The authors also suggested that, on the basis of the above findings, one would expect that those proteins which possess a particle size of approximately 35,000, or a multiple of this value, as determined by the ultracentrifugal method of Svedberg, would contain 288 amino acid residues or a multiple thereof. The fact that the stoichiometrical law given above appeared to be of general validity for all of the simple natural proteins suggested to Bergmann & Niemann that these substances were constructed on similar structural principles, and this general structural principle was summarized in the statement that every individual amino acid residue in the peptide chain of the protein molecule recurred at constant intervals. This principle, which involves the superposition of many different individual frequencies, was held to be responsible for forcing upon the structure of the protein molecule the stoichiometrical law which determines the total number of residues and the number of individual residues in the molecule.

Felix & Mager (63) have investigated the protamine clupein and claim that this substance contains 22 mols of arginine and 11 mols of monoamino acids. On the basis of this and other evidence Felix & Mager have proposed a formula for clupein in which the various amino acids are claimed to recur in a periodic manner as suggested by Bergmann [*Ann. Rev. Biochem.*, **6**, 186 (1937)]. It should be pointed out that the structure formulated by Felix, as well as the earlier structure suggested by Waldschmidt-Leitz (cf. Block, 32), are not in agreement with the requirements of the so-called "periodicity" hypothesis of Bergmann as they are not compatible with the stoichiometrical laws which govern the true "periodic" type of structure.

Coryell, Stitt & Pauling (49) have determined the magnetic moments of ferrihemoglobin and some of its compounds, and have found that in ferrihemoglobin and its fluoride the moments correspond to 5 unpaired electrons per unit of heme, indicating essentially ionic bonds. The cyanide and hydrosulfite were stated to contain one unpaired electron, i.e., essentially covalent bonds, and in the case of the hydroxide a bond of an intermediate type was indicated.

In conclusion, attention is called to two additional papers on protein structure (7, 125).

Isolation and analysis.—During the past two years an increasing number of crystalline proteins of physiological or pathological interest have been isolated by various investigators [see also *Ann. Rev. Biochem.*, 6 (1937)]. Anson (8) has reported the isolation of a crystalline carboxypolypeptidase from autolyzed beef pancreas and in addition has obtained a partially purified preparation of procarboxypolypeptidase. The crystalline carboxypolypeptidase even after repeated recrystallization retained a small amount of proteinase (3, 8), but Ågren & Hammarsten (3) claim that this impurity can be eliminated by cataphoresis in the presence of protamine. Sumner & Dounce (184) have announced, in a preliminary note, the preparation of a crystalline catalase from beef liver by fractional precipitation with dioxane, and White and coworkers (204) have isolated a crystalline protein, possessing high lactogenic activity, from the anterior lobe. Starting from a crude substance prepared from nasal mucosa, Robinson & Abraham (162), by merely recrystallizing from 0.05*N* acetic acid, obtained a crystalline lysozyme. This crystalline enzyme contained 4.4 per cent of tyrosine and 2.2 per cent of tryptophane; it was suggested that its molecular weight was probably about 18,000. Amorphous preparations of the Bence-Jones protein have been known for some time; in view of the numerous investigations conducted with this protein the isolation of a crystalline modification by Magnus-Levy (124), which is apparently identical with the older amorphous preparations, is of particular interest. Crystalline Bence-Jones protein is difficultly soluble in cold water but is soluble in salt solutions. The protein of acetaldehyde reductase has been obtained in a crystalline condition by Negelein & Wulff (142) by the fractional precipitation of Lebedew juice with heat, acetone, methanol, barium acetate and ethanol, and ammonium sulfate (see also 141).

Stanley (120, 177, 178, 180–182, 226) has continued his very interesting investigations on the plant viruses and with Loring (120, 177) has isolated a crystalline tobacco-mosaic virus protein from infected tomato plants. This crystalline virus protein and the protein previously isolated from infected tobacco plants appeared to be identical when compared in respect to their serological reactions, solubilities, sedimentation constants, optical activities, and isoelectric points. Later, Stanley (180) obtained a crystalline protein possessing the properties of the aucuba-mosaic virus, and it was concluded

that this aucuba-virus protein was different from the previously known tobacco-mosaic virus protein. This was held to be significant as it demonstrated that two different strains of the virus gave rise to two different proteins. Wyckoff, Biscoe & Stanley (226) have conducted ultracentrifugal analyses of the crystalline proteins isolated from plants belonging to different plant families and infected with different strains of tobacco-mosaic virus. The sedimentation constants of these proteins were the largest thus far found and corresponded to molecular weights (or particle sizes) of several millions, but the results of the analyses were claimed to be strictly analogous to those given by other large molecules. It was found that the sedimentation constants of these heavy particles in a given case were the same despite the fact that the samples consisted of the untreated juice of infected plants, solutions of the crystalline mass obtained by centrifuging this juice at very high speeds, and solutions of the virus isolated and purified by chemical means. It is of interest to note that heavy particles were not found in the juice of healthy plants and, furthermore, no molecules with weights as great as 30,000 could be detected in the proteins precipitated from the juice of healthy plants by ammonium sulfate. Solutions of the virus proteins recovered from tobacco, tomato, and phlox plants inoculated with the same virus strain were found to have identical sedimentation constants but it was claimed that the virus proteins of different strains consisted of a group of related but definitely different molecular species. Stanley (182) has summarized the results of the above and additional experiments by pointing out that four different strains of tobacco mosaic virus have given rise to four different crystallizable proteins of high molecular weight; it was suggested that when tobacco-mosaic virus mutates to give rise to a new strain this change is accompanied by the production of a new protein. On the other hand, the infection of plants belonging to widely different plant families with one particular strain of virus apparently always produced the same crystalline protein, but it is of interest to note that in this case the quantity of protein produced appeared to vary with the nature of the plant.

Agren & Hammarsten (3) have reported that the free amino group present in crystalline secretin can be split off without loss of physiological activity. Furthermore, when secretin was treated with aminopolypeptidase approximately 10 peptide bonds were opened (titration), but, surprisingly, the activity of the hormone was not destroyed by this drastic degradation. Secretin was not attacked by

purified carboxypolypeptidase (see above). It was claimed, furthermore, that the crystalline secretin contained 2 mols of proline, 2 mols of arginine, 1 mol of histidine, 3 mols of lysine, 1 mol of glutamic acid, and 1 mol of aspartic acid per molecule of protein.

Pappenheimer, Mueller & Cohen (151) have noted the production of a potent diphtherial toxin by growing the organisms on a medium of chemically defined composition, and Pappenheimer (152) has isolated and partially characterized a toxic protein obtained from *Corynebacterium diphtheriae* filtrates. This substance was estimated to have a molecular weight of from 14,000 to 18,000, an isoelectric point of pH 4.1, a specific rotation of -40° , and contained 16 per cent of nitrogen, 0.75 per cent of sulfur, 9 per cent of tyrosine, and 1.4 per cent of tryptophane. 0.0001 mg. of the substance sufficed to kill a 250 gm. guinea pig in five days.

Kuhn & Desnuelle (111) have reported an analysis of the protein component of the yellow ferment (190). With the exception of glutamic acid, all of the following components were determined by indirect methods. The values obtained were: arginine, 8.25 per cent; histidine, 2.75 per cent; lysine, 13.7 per cent; phenylalanine, 5.75 per cent; tyrosine, 7.75 per cent; tryptophane, 4.86 per cent; cystine, 0.34 per cent; glutamic acid, 7.1 per cent.

Kraut & Tria (109) have reopened the question as to the protein nature of pepsin [see Albers (6) for previous discussions on this point] by preparing, from Parke-Davis 1:10,000 pepsin, Northrop crystalline pepsin and Brücke pepsin (absorption on cholesterol). The Northrop pepsin contained 15.6 per cent of nitrogen and much tyrosine and tryptophane [*Ann. Rev. Biochem.*, **6**, 61 (1937)], while the Brücke pepsin was reported to contain 8.2 per cent of nitrogen and little or no tyrosine and tryptophane. Both preparations were proteolytically active.

Speakman & Townend (175) have investigated the glutamic acid and aspartic acid contents of wool and sea-gull feathers and have applied their results to the interpretation of the structure of the keratin molecule. In this case it is of interest to point out that Stakheyewa-Kavertznewa & Gavrilow (176) have questioned the molecular homogeneity of "keratin" and have fractionated wool keratin into two components (see also *Ann. Rev. Biochem.*, **5** and **6**). Block (30, 31) has investigated the amino acid content of neuroproteins and has found that the arginine-lysine ratio was practically constant despite the fact that the proteins were obtained from individuals of widely

different age groups. Calvery (39) has reported an analysis of the Type I pneumococcus specific precipitate and Cavett (40) has conducted a series of analyses on normal and goiterous human thyroglobulin. *Limulus* hemocyanin has been reported to contain approximately 17.5 per cent of nitrogen, 1.2 per cent of sulfur, 0.5 per cent of cystine, 2.8 per cent of methionine, 4.5 per cent of histidine, 6.4 per cent of arginine, and 8.9 per cent of lysine (127).

Additional analyses.—The basic and dicarboxylic amino acids of forage-grass proteins (132, 133); the arginine, histidine, lysine, tryptophane, tyrosine, and cystine content of erythrocrucioins (163); the amino acids of various protamines (61, 87, 119); the composition of hemoglobin and iodinated hemoglobin (15).

Size and stability.—The determination of the molecular weights (or particle sizes) and the pH stability regions of numerous proteins by Svedberg and his coworkers has been of great value in protein chemistry, and the reviewers wish to call attention to several articles which contain complete lists of the values that have been determined or revised during the last decade (60, 186, 187). Heidelberger & Pedersen (82) have determined the particle sizes of several antibodies and have found that horse and rabbit pneumococcus anticarbohydrate are different substances. The molecular weight of the rabbit antibody was found to be approximately the same as normal rabbit globulin (i.e., *ca.* 150,000), whereas the particle size of the horse antibody was 3 to 4 times that of the principal normal globulin component (see also 29). The same authors (81) have reported that thyroglobulin has a particle size of approximately 650,000 and an isoelectric point of pH 4.58. Wyckoff & Beard (223) have studied the pH stability regions of the Shope papilloma virus and have found that the protein splits at approximately pH 3 with the loss of physiological activity. From pH 3.3 to 7.0 the protein had a principal component with a sedimentation constant of *ca.* 260×10^{-13} cm. sec^{-1} dynes $^{-1}$ and retained its physiological activity. Above pH 7 but below pH 10 the homogeneity of the protein was not lost even though the virus activity was absent. Attention is called to the paper of Lansing & Kraemer (115) wherein it is claimed that for binary solutions changes in the partial specific volume resulting from solvation or compound formation do not affect the particle-size determinations when the sedimentation-equilibrium method is employed.

During the past year Wyckoff (220, 221, 227) has provided a new method for the isolation of proteins in developing a "quantity"

air-driven ultracentrifuge. It was found that proteins having a sedimentation constant greater than 40 could be sedimented and those with a sedimentation constant greater than 15 could be concentrated in a field not greater than 50,000 times gravity. Thus, with the aid of the quantity ultracentrifuge Wyckoff (218) has been able to prepare a concentrate of pneumococcus antibodies, and Wyckoff & Corey (219) have isolated a crystalline virus protein merely by centrifuging the sap of plants infected with tobacco-mosaic virus. The virus protein obtained in this manner was claimed to be substantially identical with that isolated by chemical means. This new technic has also been applied to the isolation of the tobacco-ring spot and other virus proteins (179), the homogeneous heavy protein from virus-induced rabbit papillomas (16), the homogeneous heavy component of tissues diseased with equine encephalomyelitis (224), and the causative agent of a chicken tumor (44).

Hydrolysis and synthesis.—Several years ago it was claimed by Ishiyama and Tazawa [*Ann. Rev. Biochem.*, **6**, 61, 285 (1937)] that pepsin was capable of splitting basic dipeptide anhydrides, and "trypsin" and papain acidic dipeptide anhydrides. Shortly after this claim was set forth a number of investigators attempted to repeat these experiments, but in every case a negative result was obtained [*Ann. Rev. Biochem.*, **6**, 61 (1937); see also reference (4)]. Recently Shibata & Tazawa (170) have reiterated the earlier claims of Ishiyama and Tazawa and, in addition, have suggested that their results are compatible with the so-called cyclol theory of protein structure (see above). The negative results of the other investigators were attributed to the use of insufficient enzyme. The reviewers find it difficult to accept the experiments of Shibata & Tazawa as they are incompatible with the general laws governing the velocity of enzymatic reactions; it is hoped that this question as to whether or not true proteinases can hydrolyze dipeptide anhydrides will be finally answered in the near future.

Flosdorf, Mudd & Flosdorf (66) have offered evidence which, if sound, invalidates the immunological experiments that have been offered in support of the view that plasteins are synthesized products having molecular dimensions approximating those of genuine proteins. Thus it becomes increasingly probable that plastein formation is a process which has little in common with the true enzymatic synthesis of proteins.

Bergmann, Fraenkel-Conrat & Niemann (23, 25) have investi-

gated the role of specificity in the enzymatic synthesis of proteins and have suggested that when the intracellular enzyme has at its disposal a number of protein fragments of different size and structure, it subjects these fragments to a series of transformations by synthesis, hydrolysis, and replacement and thereby reconstructs one peptide bond after another until there is produced a protein pattern which is stable in the presence of the enzyme. Thus it was intimated that the protein fragments available under the individual environments and the specificity of the enzyme together determine the individual pattern of the synthesized protein. Furthermore, it was pointed out that proteinases should exist which have the ability of synthesizing replicas of their own structural patterns and therefore would be able to multiply in suitable surroundings. Such a type of proteinase when placed in a suitable host organism would cause the continuous production of foreign protein. The similarity of the above property to that described by Stanley for the mosaic viruses was noted and it was suggested that it would appear desirable to investigate these and other viruses for possible proteinase activity (see also 53).

The general nature of the enzymatic degradation of proteins has been reviewed by Bergmann & Niemann (20). These authors have concluded that the first step in the degradation can be accomplished only by those enzymes that do not require a free α -amino or α -carboxyl group, i.e., the proteinases or endopeptidases. The extent of splitting of proteins by proteinases was held to be determined by the structure of the protein and the specificity of the proteinase and it was emphasized that in many cases the degradation proceeds with the formation of large amounts of amino acids.

Additional papers.—The formation of enzymes (149); the activation of partially purified pepsinogen (89); the enzymatic hydrolysis of lactalbumin (134); the action of enzymes on antibodies (165); the tyrosine metabolism of *Bombyx mori* (92); the enzymatic formation of benzamide and hippuric acid (201).

Miscellaneous.—The reaction of aromatic diazo compounds with proteins has been of great aid in introducing various groups into protein molecules and, as is well known, has been used extensively in modern immunochemistry. It has been generally assumed that the reaction involved the coupling of an aromatic diazo compound with the tyrosine and the histidine residues present in the protein molecule to form the corresponding azo compound. From the recent investigations of Eagle and his coworkers (55, 56) and of Kapeller-Adler

& Boxer (101) it is evident that the previous conception should be extended. For example, it has been found (55, 56, 101) that glycine, alanine, lysine, ornithine, phenylalanine, proline, hydroxyproline, tryptophane, and indole reacted with either diazosulfanilic acid or diazoarsanilic acid and where the reaction was restricted to a free amino group approximately 2 mols of the diazo compound were consumed (56). On the other hand, the peptide linkage, as present in diketopiperazine, the amide group of acetamide, and the imino nitrogen of arginine did not react with the diazo reagent (56). Thus, when a protein is treated with an aromatic diazo compound three different types of reaction products may be formed: i.e., azo compounds, diazoamino compounds, and bisdiazoamino compounds.

The recent clinical success of insulin-protamine compounds has led to many modifications of the basic method and analogous preparations have been made using histone (27), tannic acid (28), safranin (98), and alum (166). v. Przylecki (156, 158) has summarized the results of his investigations on the compounds formed by the union of proteins and various natural products (158) and in the case of the protein-polysaccharide compounds it was suggested that the arginine and tyrosine residues present in the protein molecule were primarily responsible for the formation of the complexes (156). Meyer, Palmer & Smyth (130) have investigated the protein complexes of chondroitin sulfuric acid and have claimed that these complexes are true salts formed in stoichiometrical proportions by the union of the basic group of the protein and the acidic group of the polysaccharide. Felix & Mager (62) have prepared a number of compounds of clupein and substances which were considered as prosthetic groups, e.g., insulin, adenine phosphate, haemin, protoporphyrin, etc. Roche & Combette (164) have shown that hemoglobin prepared from globin and protohematin has apparently the same molecular weight as the natural compound.

ADDITIONAL PROTEIN PAPERS

Glycoproteins.—The carbohydrate of lactalbumin (174); vitellomucoid and serum mucoid (150); the prosthetic group of ovomucoid (126); the proteins of egg white (229); the carbohydrate groups of various glycoproteins (102); the glycoprotein fraction from serum albumin (85); the antigenic behavior of serum proteins (86).

Denaturation of proteins.—X-ray interpretation of denaturation and the structure of the seed globulins (12); effects produced by the

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irradiation of proteins (11); the denaturation of egg albumin by ultraviolet radiation (43); the denaturation and hydration of proteins (38); the change in state of the proteins of muscle in rigor (136).

Miscellaneous.—X-ray diffraction patterns from reprecipitated connective tissue (222); X-ray diffraction patterns of crystalline tobacco-mosaic virus protein (225); the amphoteric properties of hemoglobin (47); the titration curves of oxygenated and reduced hemoglobin (72); protein films (73); the nature of antibodies (93); the isolation of immunologically pure antibody (41); the reducing groups of proteins (135); the identification of the hemagglutinin of the jack bean with concanavalin A (183); the preparation of gliadin and zein (147).

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THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF PHOSPHORUS*

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Earlier reviews on this subject by Kay (1) and by Robison (2) have appeared in the *Annual Review of Biochemistry* for 1934 and 1936.

Analytical methods.—The determination of inorganic phosphoric acid in routine biological investigations generally follows the approved colorimetric methods of Briggs (3), as well as the closely related methods of Fiske & Subbarow (4) or of Lohmann & Jendrassik (5). In recent years the transference of these methods from the colorimeter to the stuophotometer, in a variety of ways which obviate the preparation of a comparison solution, has been described (6, 7, 8). Photoelectric apparatus has been described by Yoe & Crumpler (9), and by Eddy & De Eds (10). A polarographic determination of the molybdate and phosphate ion was not successful (11). Atanasiu & Velculescu (12) have published a potentiometric phosphate determination in a solution of uranyl acetate. Pincussen & Roman (13) describe a method for the determination of phosphorus in the presence of silicon, and Pett (14) in the presence of arsenate. In Pett's method the arsenate is first reduced to arsenite by warming with bisulfite. Zinzadze (15) published a colorimetric method for the determination of phosphorus in the presence of silicon dioxide, arsenic, iron, and nitrates. According to Ammon & Hinsberg (16) ascorbic acid also can be used as a reducing agent in the determination of phosphate and arsenate. A rapid microdetermination of the phosphoric acid in water for sanitation purposes was published by Buogo (17). A new method has been described by King & Delory (18), in which a difficultly soluble precipitate is formed from phosphomolybdic acid with 8-hydroxyquinoline. The quinoline with the Folin phenol reagent produces an intense blue color, which is five to ten times stronger than that in the usual methods for phosphorus. A method for the histochemical detection of phosphate aggregates in tissue has been described by Heidermanns & Wurmbach (19).

For the determination of the total phosphate in protein-free

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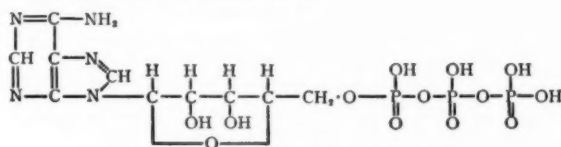
solutions, the destruction of the organic matter (containing 0.05 to 0.1 mg. phosphorus) with 1 cc. of 2 *N* sulphuric acid and for every 500 cc., 20 cc. of concentrated nitric acid, usually has been found to be sufficient. In the case of solutions containing greater amounts of organic non-volatile matter, it is recommended that hydrogen peroxide be added, or according to Gerritz (20) perchloric acid.

The colorimetric determination is based on the fact that, in general, only the inorganic phosphoric acid forms a reducible molybdenum complex with molybdic acid, while phosphoric acid esters do not react. Such esters can be analyzed either by a specific reaction of the organic component, or by a phosphorus determination after the ester has been hydrolyzed by a specific reaction. In the latter case the hydrolytic method of Lohmann (21, 22) has been shown in several instances to be adequate. In this method the rate of hydrolysis of the phosphoric acid ester is measured in 1 *N* hydrochloric acid at 100°; or in the case of esters that are hydrolyzed with some difficulty, in the vapor of boiling amyl alcohol (126°). Two phosphoric acid esters can be determined quantitatively in the presence of each other if the rate constants calculated for a monomolecular reaction differ by 10². The quantitative estimation of the triosephosphates is accomplished most simply by alkaline hydrolysis in the cold (23), and that of phosphopyruvic acid by decomposition with hypoiodite or with mercuric chloride (24). The glycerol determination of Zeisel (25) is a specific reaction for the determination of phosphorus in phosphoglycerol (in absence of free glycerol), while phosphoglyceric acid is determined by the method of Rapoport (26). Litynski (27) has published a method for determining nuclein phosphorus.

The adenylic acid system.—As a result of muscular activity, the hydrolysis and synthesis of phosphocreatine or phosphoarginine occurs simultaneously with the decomposition of carbohydrates to lactic acid, a reaction which proceeds normally in the muscle and in yeast through intermediate phosphorus compounds. In these phosphorylating and dephosphorylating processes the adenylic acid system takes the part of a carrier, since it is capable of acting as a phosphate acceptor as well as a phosphate donator. To these systems belong adenylic acid (of muscle), adenosinediphosphoric acid, and adenosinetriphosphoric acid. Adenosinetriphosphoric acid has been isolated by Lohmann (28) from octopus muscle, by Dworaczek & Barronscheen (29) from smooth muscle as the acridine salt, and by Lohmann &

Schuster (30) in larger amounts from pig's blood. At the same time molecular weight determinations carried out according to the method of Warburg, Christian & Gries (31) indicated that the adenosinetriphosphoric acid was a mononucleotide. Investigations on the occurrence of this substance in yeast may be found in the papers of Lohmann (32), Euler, Adler & Pétursson (33), and Wagner-Jauregg (34). The greatest portion of the "readily hydrolyzed" phosphate, however, is not adenosinetriphosphoric acid (32).

The following structural formula for adenosinetriphosphoric acid has been proposed by Lohmann (35).



I. Adenosinetriphosphoric acid

This structure accounts for the fact that the amino group can be converted quantitatively into hydroxyl by nitrous acid and that both hydroxyl groups in the ribose are still free, since adenosinetriphosphoric acid forms complex copper salts, and gives increased acidity in the Boeseken reaction with boric acid. According to the electrometric titration curve the adenosinetriphosphoric acid contains two acidic hydrogen atoms less than the products obtained by the hydrolytic removal of phosphoric acid. After it was found that a molecule of phosphoric acid could be removed from adenosinetriphosphoric acid (obtained from magnesium-free crab's muscle) forming adenosinediphosphoric acid (36), and that this adenosinediphosphoric acid contained one less acidic hydrogen than before the cleavage, the above structure appeared to be the only conceivable possibility. Barrenscheen & Jachimowicz (37) now state that only the difficultly hydrolyzable phosphate is removed from adenosinetriphosphoric acid by bone phosphatase, while the easily hydrolyzable phosphate remains unchanged. These results, which are not reconcilable with Lohmann's formula, were not confirmed in experiments by Lohmann & Liebknecht (38).

The role of the adenylic acid system as a phosphate acceptor and donator has been investigated thoroughly. The adenylic acid system should be designated as the phosphorylating-dephosphorylating co-ferment system, to which must still be added magnesium ions, and

which according to Lohmann is absolutely necessary for lactic acid formation and alcoholic fermentation. Whether the magnesium ion is required for *all* phosphorylating processes has not been proven.

The thorough investigation of the adenylic acid system as a phosphate carrier arose from the fact that the phosphocreatine in muscle broth or muscle extract decomposes only in the presence of adenosinetriphosphoric acid or adenylic acid (39), and that in this reaction the adenosinetriphosphoric acid becomes dephosphorylated. The adenylic acid thus formed combines with the phosphate from the phosphocreatine. In crab muscle dephosphorylation of the adenosinetriphosphoric acid results only in the formation of adenosinediphosphoric acid (36), and this partial dephosphorylation may also be the predominant reaction in vertebrate muscle. Earlier it was demonstrated qualitatively by Meyerhof & Lohmann (40) that the opposite reaction, the synthesis of phosphocreatine at the expense of adenosinetriphosphoric acid, occurs, hence these reactions are to be written as equilibria. The equilibrium: adenylic acid + 2 phosphocreatine \rightleftharpoons adenosinetriphosphoric acid + 2 creatine, is shifted far to the right in neutral solution and in the presence of dephosphorylating enzymes for adenosinetriphosphoric acid. Lohmann's assumption that the reaction is not trimolecular, but always a bimolecular reaction which proceeds through adenosinediphosphoric acid, was confirmed by an investigation of the kinetics of the reaction (41).

It was found by Lohmann & Meyerhof (24) that phosphopyruvic acid is hydrolyzed only in the presence of adenylic acid or adenosinetriphosphoric acid. Others (2, 42) observed that phosphocreatine was formed from adenylic acid, creatine, and phosphoglyceric acid or phosphopyruvic acid. The mechanism of this reaction was explained by Meyerhof & Lehmann (43, 44), Needham & Heyningen (45), and Ostern, Baranowsky & Reis (46) as follows: The adenylic acid was phosphorylated by the phosphopyruvic acid to adenosinetriphosphoric acid, and this substance acted as a phosphate carrier to the creatine. With these experiments a bridge from carbohydrate metabolism to phosphagen metabolism was created. The manner in which the phosphorylation of the carbohydrate occurs has not been fully explained. It is doubtless true that here also the adenylic acid system plays a decisive role as a phosphate carrier; thus the reaction, 2 hexose + adenosinetriphosphoric acid = 2 hexosemonophosphoric acid + adenylic acid; also: 2 hexosemonophosphoric acid + adenosinetriphosphoric acid \rightleftharpoons 2 hexosediphosphoric acid + adenylic acid.

On the other hand, according to Parnas (42), a phosphorylation of glycogen to hexosemonophosphoric acid occurs also in the absence of the adenylic acid system, in contrast to earlier experiments of Lohmann as well as more recent ones of Cori, Cori & Colowick (47, 48, 49), and of Kendal & Stickland (50). Cori, as well as Stickland (private communication), found that adenylic acid alone is effective as a phosphate carrier. It is not yet clear that here the adenylic acid is phosphorylated first to adenosinetriphosphoric acid with inorganic phosphate by the aid of a still unknown ferment system. The possibility of such a phosphorylation of the adenylic acid (or adenosinediphosphoric acid) with inorganic phosphate has been demonstrated with certainty. It occurs anaerobically in the dismutation of phosphoglyceraldehyde to phosphoglyceric acid, as Dische (51) found in red blood cells and blood hemolysates, Meyerhof *et al.* (52, 53) in yeast and muscle preparations, Schäffner & Berl (54, 55) in yeast preparations, and Needham & Pillai (56) in muscle preparations. The adenylic acid is here phosphorylated to adenosinetriphosphoric acid in a hitherto unknown coupled reaction. Monoiodoacetic acid inhibits both the dismutation and the esterification; arsenate inhibits only the esterification, while phlorhizin has no effect on either reaction (56). According to Meyerhof (53) this transference of inorganic phosphate occurs in approximately stoichiometric proportions, that is, one mol of phosphoric acid is transferred per mol of phosphoglyceric acid formed from triosephosphoric acid.

An esterification of inorganic phosphate, probably by means of the adenylic acid system, results also from the oxidative carbohydrate combustion. This is shown especially in the experiments of Runnström, Lennerstrand & Borei (57), after Lohmann as well as Engelhardt had found that aerobic conditions inhibit the decomposition of adenosinetriphosphoric acid. As in the anaerobic phosphate transfer, it is not known whether this phosphate is transferred directly by the adenylic acid system, or through another intermediate, or in what stoichiometric proportions this transfer occurs.

In some of Lohmann's earlier investigations the carbohydrate esterified was exactly equivalent to the adenosinetriphosphoric acid decomposed. As noted above Cori and Stickland find that adenylic acid also is capable of acting as a phosphate carrier in the esterification of inorganic phosphate. When one considers further that adenosinetriphosphoric acid can transfer its phosphate to carbohydrate as well as to hexosemonophosphoric acid and to creatine, and that adenylic

acid can be rephosphorylated by phosphopyruvic acid and phosphocreatine as well as anaerobically by dismutations and aerobically by inorganic phosphate, it is clear that these different possibilities, which exist together and, according to the method of preparation of the ferment, occur to different extents, cannot be pressed into a fixed scheme for the progress of the reactions in the intact muscle. This has been referred to at different times by Meyerhof (58), Parnas (42), and Lohmann (59).

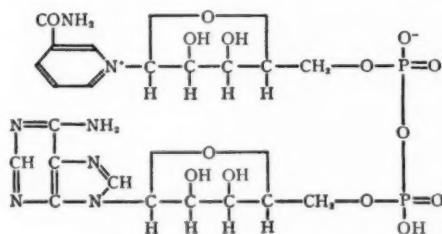
The enzymatic phosphorylation of adenylic acid and adenosinediphosphoric acid to adenosinetriphosphoric acid proceeds with great facility. But there are still some difficulties attending the chemical methods of phosphorylation. According to Ostern & Terszakowec (60) adenosine is phosphorylated enzymatically also to adenylic acid and adenosinetriphosphoric acid by fresh beer yeast or by acetone dried yeast with the addition of toluene. This reaction is not possible in muscle. Jachimowicz (61) describes the chemical phosphorylation of adenosine to muscle adenylic acid with phosphorus oxychloride in pyridine.

Several kinds of well-crystallized acridine salts of adenylic acid and adenosinetriphosphoric acid were reported by Wagner-Jauregg (62). Tipson (63) obtained the compound $C_{13}H_9N \cdot 2C_{10}H_{14}O_7N_5P$ and not the compound $C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P$ with adenylic acid under the given conditions. The results of Ferdmann (64), that inorganic pyrophosphoric acid appears in muscle, could not be confirmed either by Lohmann & Schuster (65) or by Umschweif & Gibaylo (66).

Cozymase.—After Warburg, Christian & Griesse (31) showed that the respiratory coenzyme consisted of one molecule of adenine, two molecules of carbohydrate (probably pentose), three molecules of phosphoric acid, and one molecule of nicotinic acid amide, it was found in concurrent work by Warburg & Christian (67) and by Euler, Albers & Schlenk (68, 69) that nicotinic acid amide occurred also in cozymase. The following structural formula (II) has been proposed for cozymase by Euler & Schlenk (70).

It is believed that cozymase has only one free acidic hydrogen together with a weak basic group which is due to the adenine (71). The cozymase is inactivated as a hydrogen carrier by brief treatment with a weakly alkaline solution at 100°, but it may still act as a phosphate acceptor and donator. This depends (72) on the formation of adenosinediphosphoric acid in the alkaline cleavage. The content of cozymase in yeast amounts to 0.5 gm. per kilogram of

fresh yeast according to Meyerhof & Ohlmeyer (73). The content in fresh warm-blooded muscle is about the same according to Ochoa (74). In muscle it is rapidly destroyed by autolysis (75).



II. Cozymase

According to the investigations of Euler's school, the cozymase is the hydrogen-carrying coenzyme (codehydrase I), in which the cozymase takes up two hydrogen atoms forming dihydrocozymase and then gives them up again. As such it is an absolutely necessary component in lactic acid formation and alcoholic fermentation. For this purpose Euler (76) states that two apodehydrases are required, between which the cozymase acts as an intermediate so that the transference of hydrogen from donator to acceptor is effected; that is, in alcoholic fermentation, from the donator system triosephosphoric acid-phosphoglyceric acid to the acceptor system acetaldehyde-ethyl alcohol or, in lactic acid formation, to pyruvic acid-lactic acid. In addition the dihydrocozymase can be dehydrogenated by means of the flavin-enzyme system. Nilsson (77) reports that he found cozymase also in *Azotobacter chroococcum*. Its function here has not been explained.

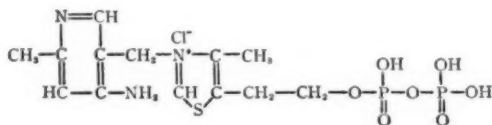
Whether the cozymase plays a role also in phosphorylating processes has not been conclusively ascertained. Euler & Adler (78) and Vestin (79) found only a limited phosphate-transferring effect with cozymase preparations, which, prepared in about the middle of 1936, were considered to be pure. On the other hand, Ohlmeyer & Ochoa (80) found that a typical phosphorylation-dephosphorylation system was dependent to a considerable extent on the ions present.

Kendal & Stickland (81) have found that very dilute muscle extract cannot be reactivated by magnesium ion + adenosinetriphosphate + cozymase, but it can be reactivated with boiled muscle extract. Hence the coenzyme system, as hitherto known, is evidently not complete.

The respiratory coenzyme.—The respiratory coenzyme (triphosphopyridinenucleotide; codehydrase II) differs from cozymase, according to Warburg, Christian & Griese, in the possession of one more molecule of phosphoric acid. They give $C_{24}H_{28}N_7P_3O_{17}$ as a molecular formula (31). A structural formula has not yet been proposed. Vestin (82) carried out an enzymatic phosphorylation of cozymase to the respiratory coenzyme with adenosinetriphosphoric acid, and Schlenk (83) a chemical phosphorylation with phosphorus oxychloride. Euler, Adler & Eriksen (84) seem to have found evidence for the reverse reaction.

According to Warburg *et al.* (85, 86) several "proteins" are necessary for the oxidative decomposition of the Robison ester.

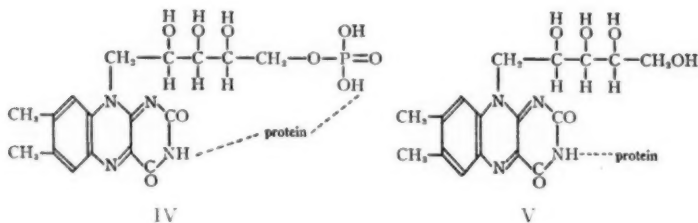
Cocarcboxylase.—Auhagen (87) found in 1932 that alkaline washed beer yeast could no longer decarboxylate pyruvic acid, and hence could not ferment sugar. This alkaline washed yeast can, however, be reactivated by treatment with boiled juice from fresh beer yeast. This thermostable principle, which was called cocarcboxylase by Auhagen, was isolated as a well-crystallized hydrochloride by Lohmann & Schuster (88, 89). It proved to be diphosphorylated vitamin B_1 , to which structure III has been given.



III. Cocarcboxylase

In the pigeon test cocarcboxylase is about twice as effective as phosphorus-free vitamin B_1 , and less effective than vitamin B_1 in Peters' catatorulin test (89, 90). A partial enzymatic synthesis by means of animal tissue has been reported by Lohmann (89), by Euler & Vestin (91), and by Tauber (92), and a chemical synthesis by Stern & Hofer (93). Several experiments of Lipmann (94) are worthy of note in that the respiration of alkaline washed *B. delbrückii*, using pyruvate as a substrate and obtaining acetic acid and carbon dioxide as products, is increased by cocarcboxylase, and a still greater increase occurs with cocarcboxylase + phosphoflavin. The decarboxylation occurs therefore in this case only with simultaneous dehydrogenation. Vitamin B_1 was without effect.

The behavior of cocarboxylase in enzyme investigations indicates that vitamin B₁, as well as vitamin B₂, which cannot be synthesized by the body, probably acts as the prosthetic group of an enzyme. Nicotinic acid has been shown to be the curative agent in pellagra. Experiments in search of further relationships should give valuable information. For vitamins B₁ and B₂ it is characteristic that a typical enzyme reaction is really given only by the phosphorylated compound—less so by the phosphorus-free. According to Kuhn & Rudy (95) lactoflavin and lactoflavinphosphoric acid are effective as coenzymes in the system of Warburg & Christian: Neuberg ester + coenzyme from blood cells + "intermediate enzyme" from yeast + lactoflavin-protein + increasing amounts of lactoflavin or lactoflavinphosphoric acid. The activity of the lactoflavinphosphoric acid is, however, several hundred times greater than that of lactoflavinic acid. Kuhn & Rudy explain this behavior on the basis that there are two different ways of combining the active substance, lactoflavin, with the protein, both of which lead to catalytically active symplexes in the sense of Willstätter & Rohdewald (96). The phosphorus-free and the phosphorus-containing flavin enzyme differ essentially in the stability of the union of the prosthetic group with the protein carrier. Lactoflavin is held much less strongly by the carrier than the lactoflavin-5-phosphoric acid. The equilibrium: prosthetic group + protein \rightleftharpoons symplex is displaced much farther to the right with lactoflavinphosphoric acid than with lactoflavin. Lactoflavin can be removed from the compound lactoflavin-protein by dialysis at pH 7, while Theorell (97) finds that lactoflavinphosphoric acid can be dialyzed from the "yellow enzyme" only in acid solutions at pH > 4. The protein carrier seems to bind intimately the phosphoric acid group in some way upon which the union of the prosthetic group to the carrier is dependent. On the other hand in the "yellow enzyme" still other groups in the lactoflavin molecule take part in establishing the protein-prosthetic group bond. The most probable is the imide group in the 3-position (98, 99), because upon methylation of this group both enzymatic and vitamin activities disappear. The vitamin activity of the phosphorus-free compounds may be attributed to the fact that they are phosphorylated in the body, as was demonstrated by Rudy & Kuhn (100) in the esterification of lactoflavin with the aid of intestinal mucosa. Kuhn & Rudy (95) indicate the prosthetic group-protein bonds in "yellow enzyme" (flavo-phospho-protein) and in flavo-protein as in formulas IV and V.



In addition the authors state that in the flavin-enzyme which is produced in the absence of phosphoric acid, the essential point of connection is the imide group in the 3-position. Since, however, araboflavin is in all probability inactive, it must be assumed that the entire prosthetic group is imbedded in the protein molecule.

Guanidinophosphoric acids.—Argininephosphoric acid has been isolated from octopus muscle by Lohmann (28). Rosenfeld & Bagdassarjan (101) report that phosphocreatine occurs to the extent of about 7 to 16 mg. per cent in the skeletal muscles of marine teleosts (*Acanthopterygii* and *Lophobranchii*), while Bagdassarjan (102) found that in addition to phosphocreatine still another substance occurred in the muscle of sharks and of two species of ray. This substance was easily hydrolyzed by cold dilute acid and was considered to be argininephosphoric acid. This recalls earlier work (103) on the simultaneous occurrence of phosphocreatine and argininephosphoric acid in the jaw muscles of *Strongylocentrotus lividus* and in the body of the Enteropneust, *Balanoglossus*, while, according to Eggleton as well as Meyerhof, only phosphocreatine occurs in *Amphioxus*. The findings of Bagdassarjan are, hence, surprising. Zilinski (104) reports that one third of the creatine present in frog's eggs exists as phosphocreatine.

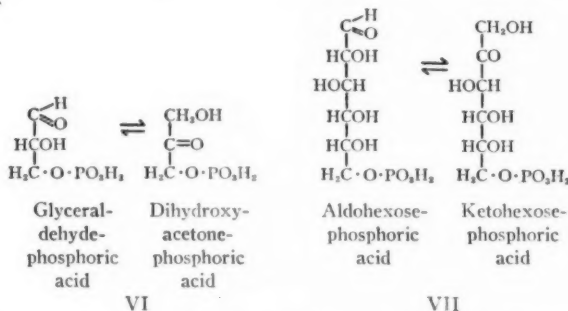
As previously stated, creatine in a properly prepared muscle extract is converted to phosphocreatine by adenosinetriphosphoric acid, and adenylic acid to adenosinetriphosphoric acid by phosphopyruvic acid (or phosphoglyceric acid). These reactions were worked out by Lehmann (44) according to a method which is readily applicable to the preparation of phosphocreatine and argininephosphoric acid. Meyerhof, Schulz & Schuster (105) proceed directly from hexosediphosphoric acid and pyruvic acid, whereby all the phosphate of the hexosediphosphoric acid is transferred at one time to creatine through the agency of adenosinetriphosphoric acid, and in addition an equivalent

amount of inorganic phosphate becomes esterified with adenylic acid in the dismutation of the triosephosphoric acid.

The metabolism of guanidinophosphoric acid in organ activity has been frequently investigated, for example the work of Engel & Gerard (106) on the metabolism of inorganic phosphate, argininephosphate, adenosinetriphosphate, difficultly hydrolyzable and total phosphate.

1-Phospho-2-keto-compounds.—Embden, Deuticke & Kraft (107) have, in their well-known scheme for lactic acid formation, assumed the splitting of hexosediphosphoric acid into one molecule each of glyceraldehydephosphoric acid and dihydroxyacetonephosphoric acid.

Meyerhof & Lohmann (108) then demonstrated that this cleavage in a coenzyme-free muscle extract occurs as an equilibrium, the heat effect of which can be thermodynamically calculated from the variation in concentrations with the temperature (109). Dihydroxyacetonephosphoric acid was practically the only triosephosphate product of this reaction under the conditions of the experiment, with glyceraldehydephosphoric acid produced only in traces. This behavior depends on the enzymatic conversion of the glyceraldehydephosphoric acid to dihydroxyacetonephosphoric acid (VI). Some time ago an enzymatic equilibrium between aldohexosemonophosphoric acid and ketohexosemonophosphoric acid (VII) had been found by Lohmann (110).

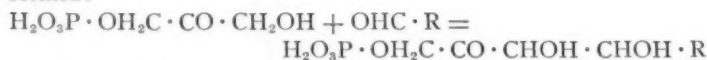


The composition at equilibrium in the case of the hexosemonophosphoric acid is about 75 per cent aldosephosphoric acid and about 25 per cent ketosephosphoric acid. The naturally occurring 6-hexosemonophosphoric acids produced in lactic acid formation and alcoholic fermentation in yeast maceration juice are therefore "equilibrium esters." The hexosemonophosphoric acid which has been isolated from fermenting yeast maceration juice is, according to Smythe

(111), contaminated with glycerolphosphoric acid. Smythe obtained a crystalline calcium salt which was an equimolecular mixture of calcium hexosemonophosphate and calcium glycerolphosphate.

The enzyme which brings about a rearrangement of the carbonyl group, and which, according to Lohmann, occurred in all the organisms investigated, and also in yeast, and according to Tankó & Robison (112) in bones was designated by Robison as "phosphohexomutase." Since both triosephosphoric acids are also rearranged in a similar manner with the equilibrium displaced predominantly towards the side of the keto compound, this enzyme, or group of enzymes, should be designated as "oxo-isomerases" (59). The enzyme affects only the phosphorylated compounds and not the phosphorus-free substances.

The cleavage of hexosediphosphoric acid into two molecules of triosephosphoric acid, is the reverse of the synthesis of hexosediphosphoric acid from both components. The enzyme causing this equilibrium to take place has been called "zymohexase" (23). This reaction was shown by Meyerhof, Lohmann & Schuster (113) to be an aldol condensation between the dihydroxyacetonephosphoric acid and an aldehyde, as a result of which keto-1-phosphoric acid esters are formed:



The reactions of dihydroxyacetonephosphoric acid with acetaldehyde and with glyceraldehyde in which a methyltetrosephosphoric acid or a fructose-1-phosphoric acid is formed are described in more detail. Fructose-1-phosphoric acid had already been obtained by Robison and associates (114, 112) through partial dephosphorylation of the hexosediphosphoric acid with bone phosphatase. In the condensation of optically inactive dihydroxyacetonephosphoric acid with acetaldehyde, a laevorotatory methyltetrosephosphoric acid is formed and condensation with *d*-glyceraldehyde gives *d*-fructose-1-phosphoric acid. Condensation with racemic glyceraldehyde gives also *l*-sorbitol-1-phosphoric acid as a result of the reaction with the *l*-glyceraldehyde. Other enzymatic condensation products were obtained with formaldehyde, glycolic aldehyde, propionaldehyde, α -hydroxypropionaldehyde, methylglyoxal, butyraldehyde, β -phenylpropionaldehyde, and aldol (115). All of these reactions are equilibrium reactions. In an actual experiment one begins with an inactivated or dialyzed muscle extract and hexosediphosphoric acid which will be hydrolyzed to dihydroxy-

acetonephosphoric acid. A large excess of aldehyde is used in order to displace the equilibrium as far towards the side of synthesis as possible. It is assumed that all these reactions are catalyzed by the same enzyme, "aldolase," which is capable of contacting the substrate at two points:



The first point of contact is assumed to be on the entire molecule of dihydroxyacetonephosphoric acid and the second only on the aldehyde group. The rest of the aldehyde molecule seems to influence only the reaction velocity and the position of equilibrium. It is noteworthy that those aldehydes do not react which have a strong negative group in the immediate neighborhood of the aldehyde group, for instance trichloroacetaldehyde or benzaldehyde in contrast to phenylpropionaldehyde. The relationships here are very similar to those which occur in the enzymatic cleavage of synthetic dipeptides.

It has further been shown that all those keto-1-phosphoric acid esters, which are not capable of forming a furan or pyran ring in their molecule, like dihydroxyacetonephosphoric acid, tetrosephosphoric acid, and methyl or ethyl tetrosephosphoric acid, are quantitatively hydrolyzed even by cold dilute alkali (e.g., in 1 *N* NaOH at 20° in ten minutes). In contrast, compounds such as keto-1-hexosephosphoric acid are stable in dilute alkali.

The biological importance of the enzyme aldolase probably lies in the scission (or synthesis) of hexosediphosphoric acid into dihydroxyacetonephosphoric acid and glyceraldehydephosphoric acid. This reaction with the enzyme oxoisomerase may be written as follows: Hexosediphosphoric acid \rightleftharpoons dihydroxyacetonephosphoric acid +

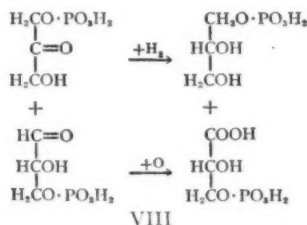
glyceraldehydephosphoric acid.

Glyceraldehydephosphoric acid \rightleftharpoons dihydroxyacetonephosphoric acid.

The dotted arrow indicates that the second reaction is displaced well to the side of the dihydroxyacetonephosphoric acid (approximately 95 per cent).

According to the Embden-Meyerhof scheme dihydroxyacetonephosphoric acid and glyceraldehydephosphoric acid react with each other in an oxidation-reduction reaction in which 3-phosphoglyceric acid and α -glycerolphosphoric acid are formed. From the investigations of Fischer & Baer (116) it was demonstrated that the α -glycerolphosphoric acid arising in lactic acid formation did not belong to the

d-glyceraldehyde series but to the *l* series, since by phosphorylation of the free hydroxyl group in *d*-acetoneglycerol, *l*-glycerolphosphoric acid can be obtained. On the other hand *d*-glyceric acid was produced by the oxidation of *d*-glyceraldehyde, which by phosphorylation should be converted to 3(—)-phosphoglyceric acid. The natural α -glycerolphosphoric acid therefore can arise only from the reduction of the dihydroxyacetonephosphoric acid. In confirmation of these results Kiessling & Schuster (117) obtained by the oxidation of natural α -glycerolphosphoric acid not the biological 3(—)-phosphoglyceric acid but the enzymatically inactive 3(+)-phosphoglyceric acid. One can write, then, the dismutation of the triosephosphoric acids according to VIII.



Mawson (118) has reported that hexosediphosphoric acid can be converted to lactic acid by a way different from that indicated above, using muscle extract warmed to 50° to 55° in which only glutathione is required as the coenzyme. Needham & Lehmann (119) explain this by the conversion of the triosephosphoric acid to methylglyoxal non-enzymatically, which is then converted enzymatically into lactic acid by methylglyoxalase with glutathione acting as the coenzyme.

Glucose-1-phosphoric acid.—Cori, Cori & Colowick (47, 48, 49) have found that in leached muscle broth and also in dialyzed magnesium-free muscle extract in the presence of inorganic phosphate, glycogen, and small amounts of adenylic acid, the glycogen is first esterified to glucose-1-phosphoric acid, which is converted enzymatically, and very rapidly, in the presence of magnesium ions into 6-hexosemonophosphoric acid (an equilibrium ester). The same compound was synthetically prepared by phosphorylation of α -1-bromotetraacetyl-glucose with silver phosphate, and then deacetylation and cleavage in cold dilute hydrochloric acid. Glucose-1-phosphoric acid is considered as an α -glucopyranose-1-phosphoric acid. The compound has no reducing power because it contains no free gluco-

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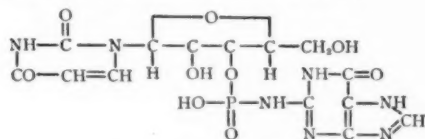
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sidic hydroxyl group. The phosphoric acid can be removed in 1 *N* H₂SO₄ at 100° in ten minutes, yielding glucose. The specific rotation of the barium salt is $[\alpha]^{25}_D = +75^\circ$, and for the free acid it is $+120^\circ$. This ester of Cori may be considered as a natural intermediate product in the esterification of the carbohydrate to hexosediphosphoric acid.

Aminoethylphosphoric acid.—An aminoethylphosphoric acid ester is described by Outhouse (120), which occurs in especially large amounts in malignant tumors, and which seems to be specific for these tumors. Malignant tumors contain about 36 mg. per cent, while the pancreas, liver, embryo (cattle), placenta, and benign tumors contain about 1 mg. per cent.

A synthesis of aminoethylphosphoric acid and cholinphosphoric acid was reported by Plimmer & Burch (121). They may be prepared by treating the base with phosphorus oxychloride, or by the action of phosphoric acid and phosphorus pentoxide on the bases, or of ammonia or trimethylamine on chloroethylphosphorus oxychloride. The substances are not hydrolyzed by dilute alkali at 100°, and are acted upon only slowly by 1 *N* HCl at that temperature.

Concerning phosphorus compounds in plants, the work of Haas & Russel-Wels (122) on marine algae, of Potop (123) on wheat leaves, of Posternak (124), and Tychowski & Masior (125) on the phosphorus of starch, should be mentioned. Bredereck & Richter (126) have developed a new conception of the structure of yeast nucleic acid. They obtained by partial hydrolysis guanineuracilic acid, in which the guanine is united through its amino group to the phosphoric acid residue of the uracilic acid (IX).



IX. Guanineuracilic acid

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CARBOHYDRATE METABOLISM*

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ENDOCRINE AND NEURAL REGULATION OF CARBOHYDRATE METABOLISM

The investigations of the endocrine and neural regulation of carbohydrate metabolism during the past year may be classified under three headings: oxidation, glycogenesis, and glycogenolysis. The first of these includes a study of diminished ability for the oxidation of carbohydrate in most tissues of the body following pancreatectomy, a diminution ameliorated by extirpation of the anterior pituitary or the adrenal cortex; the second, impaired glycogenesis caused chiefly by the removal of the hypophysis or the adrenal cortex; and finally, the condition of increased glycogenolysis frequently originating in the central nervous system and mediated by the adrenal medulla.

Diabetes may have more than one origin. No longer is it necessarily considered a condition of simple insulin deficiency, for human diabetes occurs frequently with no demonstrable changes in the pancreas. The present view includes rather the influence of the various endocrine glands in the etiology of diabetes. This conception has been demonstrated by the effect of hypophysectomy on the depancreatized dog (1), and of adrenalectomy on the depancreatized cat (2). The significance of the endocrine balance is seen again in the observations of Lukens (3) and Collip, Selye & Neufeld (4), and Greeley (5), who note different reactions of various species to pancreatectomy. Depancreatized pigs, monkeys, and rabbits suffer a mild diabetes and therefore respond in the same manner as do dogs and cats that are not only depancreatized, but also are either hypophysectomized or adrenalectomized.

Perhaps the first question to be decided is whether the depancreatized animal is capable of oxidizing carbohydrate. Despite a respiratory quotient of 0.7, not increased by the ingestion of carbohydrate, and a quotient of 0.7 for excised renal and muscle tissues (6), the brain still maintains a respiratory quotient of unity after pancrea-

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tectomy (7). The brain, if no other tissue, oxidizes carbohydrate during diabetes. Furthermore, the limited ability of the diabetic organism to oxidize carbohydrate may be heightened under certain conditions. For example, Barker, Chambers & Dann (8) observed an increased combustion of carbohydrate in the depancreatized animal subjected to prolonged fasting. Although no tissues of these animals were studied *in vitro*, it is suggestive that Shorr (9), using depancreatized animals, noted a rise in the respiratory quotient of muscle allowed to respire from five to ten hours in the Warburg apparatus. Thus, it appears that the insulin increases, but is not essential for the oxidation of carbohydrate.

The prolongation of life in the depancreatized animal by hypophysectomy or adrenalectomy raises the question as to whether the effect of these additional extirpations is to increase the oxidation of carbohydrate. Hypophysectomy may eliminate an anti-insulin action, as seen by the lowered D: N ratio, a phenomenon indicating increased combustion of carbohydrate. This is further disclosed in Houssay animals by the higher basal respiratory quotients, as well as by a demonstrable oxidation of a certain amount (15 gm.) of ingested carbohydrate in three of twelve animals (10). The respiratory quotient of muscle excised from the Houssay animal, in contrast with muscle taken from a depancreatized one, indicates the oxidation of some carbohydrate (11). Nevertheless, in Houssay animals as well as in depancreatized ones, the ability to oxidize carbohydrate must vary among organs, since excised renal tissue retains a respiratory quotient of 0.7 (12). These *in vitro* observations indicate that hypophysectomy does not increase the oxidation of carbohydrate in the diabetic organism to normal levels. The same conclusion is attained by *in vivo* experiments; Reid (13) observed a diminished utilization of carbohydrate in hypophysectomized, depancreatized cats in comparison with normal ones.

In animals not depancreatized it has also been shown that the anterior pituitary gland diminishes the oxidation of carbohydrate. Such an action has been demonstrated with extracts of this gland by Meyer, Wade & Cori (14), and by Russell (15). This effect may be lost if the injections are continued from two to three weeks (16). Young (17), on the other hand, was able to produce a permanent glycosuria and ketonuria by the injection of increasing amounts of extracts of the anterior pituitary gland. It seems that an intrinsic ability of the tissues to oxidize carbohydrate is under the influence

of an endocrine balance, increased by insulin and diminished by the anterior pituitary, or, as will be subsequently discussed, the adrenal cortex.

The prolongation of life of the depancreatized animal subjected to additional extirpations may involve factors other than increased oxidation of carbohydrate: the diminished protein metabolism (1), the lessened ketosis and other changes of fat metabolism (18, 19), as well as the lowered metabolic rate (20), all contribute to this end. Important in this connection is the diminished glycogenesis which, with decreased protein metabolism, presents less carbohydrate for the maintenance of blood sugar, thus serving to ameliorate hyperglycemia and glycosuria in post-absorptive conditions (21). The effect of the anterior pituitary in glycogenesis has been studied by injections of extracts, implantations of the anterior pituitary and by extirpation of that gland. The injection of hypophyseal extracts increases liver and muscle glycogen of rabbits (22). Implantations of this gland restore to normal values the diminished muscle glycogen of hypophysectomized toads (23). Extirpation, moreover, is followed by depletion of liver glycogen in fasted rats (24), and dogs (25). An exception is found in neoplastic tissue, which, in hypophysectomized animals, contains significantly more glycogen than in the controls. These cells are, therefore, as autonomous in their carbohydrate metabolism as they are in their growth (26).

The diminished glycogenic ability of hypophysectomized animals may explain in part the observations of Cope (27) and Cope & Thompson (28) that although injections of adrenaline and insulin increase the concentration of blood lactic acid, they produce less storage of liver glycogen than they do in the normal animal. These authors conclude that the utilization of lactic acid by the liver is impaired, another sign of diminished glycogenesis.

Since hepatic glycogenesis is one of the factors influencing the concentration of blood glucose, it is possible to study this function of the liver with the aid of glucose-tolerance curves. A low blood sugar is frequently observed in post-absorptive hypophysectomized animals. Nevertheless, Samuels & Ball (29) noted the diabetic type of tolerance curve following the injection of glucose in rats hypophysectomized five weeks. A similarly impaired carbohydrate tolerance was also reported by Russell & Cori (30) on intravenous infusion of glucose. Corey (31) noted that injections of cortin help to maintain the level of the blood sugar of the fed hypophysectomized rat. On

the other hand, Samuels, Schott & Ball (32) recorded that the prolonged glucose-tolerance curve of fasted hypophysectomized rats was not improved by injections of cortin. This is in contrast with adrenalectomized animals in whom these injections did increase the utilization of glucose.

Glycogenolysis, in contrast with glycogenesis, may remain unimpaired after extirpation of the pituitary gland. Russell & Cori (30) observed that the intravenous injection of adrenaline in hypophysectomized rats produced an increase of blood sugar similar to that occurring in normal controls. These workers suggest that the lessened effect on blood sugar when adrenaline is administered subcutaneously is due to a slower rate of absorption of epinephrine from subcutaneous tissues in the absence of the hypophysis.

Parkins, Hayes & Swingle (33) believe that the adrenal gland is not directly concerned with the metabolism of carbohydrate because adrenalectomized dogs, except in terminal collapse, show no significant deviations of blood sugar from the normal. Moreover, removal of the entire adrenal gland does not prevent the re-establishment of normal blood sugar values after insulin hypoglycemia (34). Britton & Silvette (35), however, emphasize the profound fall of carbohydrate stores of the body after adrenalectomy. Long (2) postulates a carbohydrate-regulating hormone in the cortex of the adrenal gland, a hormone not present in great amounts in cortical extracts now used, for adrenalectomized depancreatized animals receiving these extracts exhibit an ameliorated form of diabetes similar to that of the hypophysectomized animal, characterized among other changes by a lower D:N ratio and a lesser ketosis. Perhaps an excess of such a hormone occurring in a patient with hypercortico-adrenal syndrome is the cause of the diabetic condition observed in such cases (36). A new method to diminish the severity of the diabetes following pancreatectomy has been developed by Fazekas, Himwich & Martin (37), who demonstrated that ligation of the lumbo-adrenal veins prolongs the life of depancreatized cats and dogs receiving neither insulin, cortin, nor salt therapy. Most investigators agree that the cortex of the adrenal gland exerts an important influence on carbohydrate metabolism.

Since the removal either of the anterior pituitary or of the adrenal cortex ameliorates diabetes, it is important to determine whether they exert their influence independently of each other. An interrelationship between these glands is indicated by the degeneration of the adrenal

cortex after hypophysectomy. Long (2) injected anterior pituitary extracts into partially depancreatized adrenalectomized rats and did not obtain a diabetogenic effect. Similarly, prolactin-adrenotropic (closely allied to the diabetogenic hormone) provoked glycosuria in hypophysectomized, but not in adrenalectomized cats, indicating that the anterior pituitary may function *via* the adrenal cortex. In contrast, Samuels, Schott & Ball (32) conclude that the decreased ability of the hypophysectomized rat to remove excess glucose from the blood is not primarily dependent on the adrenal cortex. Houssay & Leloir (38) have demonstrated, moreover, that excision of the adrenal gland does not reduce the hyperglycemia caused by injection of hypophyseal extracts in dogs, while implantation of anterior pituitary glands increases the diabetes of depancreatized adrenalectomized toads (39). Thus, today, it is impossible to decide whether the anterior pituitary and adrenal cortex each contains carbohydrate-regulating hormones, or whether the removal of either of these glands exerts its effect by causing subsequent degeneration of the remaining gland. One of the difficulties in the solution of this problem lies in the fact that the functions of each gland are not limited to the regulation of carbohydrate metabolism and that many other changes following the removal of either gland secondarily influence carbohydrate metabolism. However, it is possible to conclude from the observations of Bennett (40) on hypophysectomized rats that the anterior pituitary gland may affect glycogenesis directly, as well as through the adrenal cortex, for both simple alkaline extract and the adrenocorticotrophic hormones are glycogenic in action.

The importance of the brain in the regulation of carbohydrate metabolism has been emphasized by Vonderahe (42) who observed cerebral lesions in some patients with hyperglycemia and glycosuria. This author, therefore, proposes a central origin for certain types of diabetes. "Such a site may include the original point of stimulation for the mobilization of liver glycogen, causing the increase of blood sugar after morphine. In the chronic spinal cat, morphine cannot discharge the sympatheticoadrenal system because of interference with the paths connecting the higher centers with the adrenal medulla and the sympathetic nerves" (43, 44). Himwich & Fazekas (45) found that insulin resistance during infection is similarly of central origin and disappears after denervation of the adrenal gland. In the emergency of infection adrenaline is liberated even at high levels of blood sugar. In normal animals, however, this anti-insulin mechanism

begins to operate only during hypoglycemia (46). This increased production of adrenaline occurring during hypoglycemia is probably of central origin, too, for LaBarre & Saric (47) record that perfusion of the cerebral region of a dog with hypoglycemic blood produces a reflex hyperadrenalemia. Looney & Cameron (48) have observed decreased glucose tolerance in schizophrenic patients after prolonged insulin therapy. These observers concluded that this treatment develops greater activity of the neuro-endocrine mechanisms. Another possibility is one of diminished insulin secretion under these conditions. Variations in insulin sensitivity therefore depend upon a complicated process including a central factor.

LaBarre & Kettenmeyer (49) have found that post-insulin hyperglycemia is inhibited by sodium barbital, and believe this action is due to the paralytic effect on thalamic centers, sensitive to the glucose level of the blood. However, in addition, there may be a peripheral action, since Hrubetz & Blackberg (50) report that injections of adrenaline produce only a moderate hyperglycemia in cats narcotized with pentobarbital. They attribute this result to a direct depressant influence of the barbiturate on liver glycogenolysis. In describing the phenomenon of post-insulin hyperglycemia, Somogyi *et al.* (51) emphasize the intrinsic functions of the liver with a preponderance of glycogenolysis over glycogenesis. In view of the fact that Soskin *et al.* (52) stressed rightfully these intrinsic hepatic mechanisms, it seems appropriate to attempt an evaluation of the relative importance of the glandular and central influences on the liver. From experiments on hypophysectomized adrenalectomized cats with denervated livers, it has been inferred (53) that this organ may function in the absence of its assumed humoral and nervous control. These animals retained at least one adrenal cortex, as well as the pancreas. However, certain functions ascribed chiefly to the liver may occur in eviscerated animals. Bergman & Drury (54) have noted a utilization of glucose more rapid in the fed than in the fasted eviscerated animal. Thus, the response to previous ingestion of carbohydrate is not limited to the action of the liver, but is more generalized and, according to these authors, is to be attributed to the effects of various glands of internal secretion on the tissues. The evidence reviewed in this section presents eloquent testimony to the influence of hormonal and neural mechanisms on the rates of glycogenesis and of glycogenolysis. These extra-hepatic effects constitute a factor of prime importance. The intrinsic hepatic mechanisms for glycogenesis and glycogenolysis,

therefore, are under the homeostatic regulation of the endocrine glands and of the nervous system.

BLOOD SUGAR

The levels of blood glucose and the glucose-tolerance curves are of diagnostic importance for they are resultants of the various factors regulating carbohydrate metabolism. This equilibrium is disturbed, according to McCance (55), in human subjects maintained on salt-deficient diets, who exhibit impaired glucose tolerance. The sweeping out of sodium salts which Adlersberg & Wachstein (56) noted after pancreatectomy, therefore, intensifies the diminished glucose tolerance which occurs, in any case, with this condition. McQuarrie (57) observed the beneficial effects of sodium salts in diabetic children, but potassium salts increased glycosuria. Silvette & Britton (58) demonstrated that potassium acetate raises blood sugar and diminishes liver glycogen.

Wilder & Wilbur (59) and Gould, Altschuler & Mellen (60) report favorably on the use of the two-dose glucose-tolerance test (61). It is a more accurate means of differentiating between the diabetic and the non-diabetic patient than is the one-dose test now in general use; the second dose reveals an increased glucose tolerance in the normal subject, but not in the diabetic. Employing both the one- and the two-dose tests, Deren (62) disclosed impaired tolerance in 50 patients over 55 years of age. Fletcher & Waters (63) noted that glucose tolerance was improved markedly by the administration of small amounts of fructose.

INSULIN

Before examining the effects of the injection of insulin it might be well to consider the insulin requirement of the body. Using depancreatized dogs Greeley (64) observed that the amount of insulin required to maintain normal levels of blood sugar varies between 0.005 and 0.035 units per kg. per hr. Presumably this is the normal requirement. Strack & Balzer (65) found that 0.005 units per kg. per hr. caused no fall in blood sugar in normal fasting animals; that 0.01–0.025 units per kg. per hr. diminished blood sugar to the lower limits of normal fasting values, while with 0.05 units, blood sugar fell to less than 50 mg. per cent. Comparable doses of insulin, therefore, produce similar effects both in the depancreatized and in the

intact animal. Perhaps the injection of insulin diminishes the secretion of endogenous insulin.

Hypoglycemic convulsions.—Various studies have been made concerning the mechanism of insulin convulsions. Drabkin & Ravdin (66) have shown that in well hydrated dogs hypoglycemic convulsions are more easily precipitated than in dehydrated animals. The sequence of events leading to convulsions in hyperinsulinism, according to these authors, is severe hypoglycemia \rightarrow anhydremia \rightarrow rise of cerebrospinal fluid pressure to critical levels \rightarrow convulsions. The observations of these authors that stellate ganglionectomy prevents insulin convulsions have not been substantiated by Phillips & Barker (67). Corwin (68) found that thirty per cent of his dogs lapsed into coma without typical convulsions. The remaining animals became increasingly susceptible to convulsions on the second and third days of insulin injections; after this time the susceptibility became stabilized. Kerr and associates (69, 70) have been able to correlate hypoglycemic convulsions with the level of cerebral glycogen. Although stable under a wide variety of conditions, cerebral glycogen is nevertheless diminished after injections of insulin. In the normal dog, the glycogen content of the brain averages about 100 mg. per cent, and in the cat and rabbit it is somewhat lower. When the brain glycogen reaches levels below 50 mg. per cent, hypoglycemic symptoms develop. Prostration and incoordination appear in cats with intact adrenals, but convulsions occur in adrenal-inactivated cats. The free glucose of the brain also diminishes at this time. Georgi (71) believes that hypoglycemic convulsions are precipitated by two consequences of the injection of insulin: the first, an alkalosis, and the second, the development of an electrical potential on the surface of the ganglion cells as a result of differences of concentration of glucose between extracellular fluids and the cell. The degree of hydration, the increased pH, the levels of cerebral glycogen, and of sugar are all factors to be considered in the explanation of hypoglycemic convulsions.

If the glycogen concentration of the brain is regarded as a result of a dynamic equilibrium, then the fall in glycogen observed by Kerr and coworkers must be due to the fact that the balance between the amount of glycogen formed and the portion utilized is changed. From the results of Himwich & Fazekas (72) it is apparent that less blood glucose is absorbed for the maintenance of cerebral glycogen, although it is possible that more carbohydrate is oxidized by the brain during insulin hypoglycemia. These workers conclude that the

diminution of oxygen consumption of the canine brain is secondary to lack of substrate and that the chief foodstuff of the brain is carbohydrate. In contrast with brain, muscle, which can oxidize fat, revealed no significant change in the utilization of oxygen during hypoglycemia. Himwich, Bowman, Wortis & Fazekas (73) also observed a depression of cerebral metabolism in schizophrenic patients receiving the insulin treatment. These workers were able to correlate the neurological and mental changes with the diminished cerebral utilization of oxygen. The responses to a moderate depression of oxygen uptake were those of stimulation and release, as characterized by increased motor activity, while a more marked depression of cerebral metabolism was accompanied by coma. The mechanism of heightened gastric motility following the injection of insulin is attributed by Lalich, Youmans & Meek (74) to the augmenting action of the vagal centers. This may be a visceral expression of the stimulation occurring with a moderate depression of cerebral oxidation.

The increasing frequency of recognition of hyperinsulinism, whether due to endogenous or injected insulin, has led to renewed interest in the study of this syndrome. Sherrill & MacKay (75) have noted that after insulin shock has been maintained for 24 hours or longer, it is impossible to resuscitate animals despite injections of glucose. The irreversible changes which occur during prolonged hypoglycemia might be imputed to the depletion of the small amount of cerebral glycogen, thus depriving the brain of its carbohydrate stores and therefore permitting the cerebral oxygen uptake to fall to critical levels. Morphological evidence for the maintenance of cerebral integrity during short periods of hypoglycemia is seen in experiments on rabbits by Baker & Lufkin (76), who observed no cellular changes of a pathological character in the brain. These short periods are comparable with those occurring in patients with schizophrenia receiving insulin therapy. In view of the excellent physical condition of these patients during the treatment, it is of interest that insulin increases the retention of amino acids by the tissues (77), and that protamine insulin is suggested by MacKay & Callaway (78) as a means of improving alimentation. In the past year the use of insulin in the treatment of schizophrenia has become widespread. For that reason the reference of a symposium on this subject is included (79).

Insulin compounds.—A vast amount of literature has appeared on the use of protamine insulin and its comparison with standard insulin. Bischoff & Jemtegaard (80) find that by injecting insulin in

divided doses at regular intervals it has been possible to reproduce hypoglycemic curves resembling those obtained by injecting insoluble insulin combinations; hourly doses of 1/24 unit of insulin per kilo or less still produce hypoglycemia. These observations bring out more strongly the advantages of protamine insulin—more continuous absorption and reduction of the number of daily injections (81, 82). On the other hand, the more intense action of standard insulin, as well as its shorter duration, are preferable during conditions of increased insulin resistance, especially when accompanied by rapid changes in the condition of the patient occurring during infection (45, 83). Since the original observation of Hagedorn (84) that protamine delays the absorption of insulin, many studies have been made on the rates of action of various insulin products: alum-precipitated insulin (85), spermine, zinc and insulin (86), histone insulin (87, 88), insulin tannate (89), the effects of copper, iron, zinc, and reduction of insulin (90, 91), as well as of the attenuation of insulin action by interfacial adsorption (92). Further studies made on the administration of insulin by routes other than subcutaneous reveal its successful absorption from the stomach (93), intestinal loops (94), and skin (95). Thus, new preparations of insulin are being administered by various routes in many conditions in addition to diabetes.

GLYCOGEN

Glycogen formers.—Observations of glycogenic substances include fatty acids, amino acids, carbohydrates, and carbohydrate split products. The increases of liver glycogen after the administration of triglycerides of fatty acids containing an even number of carbon atoms could be accounted for by the glycerol fraction. On the other hand, after feeding with triglycerides of fatty acids containing an odd number of carbon atoms, much larger amounts of glycogen were formed than could have originated from the glycerol moiety of the fat (96). Similar results were obtained with the ethyl esters, only those containing fatty acids with an odd number of carbon atoms being glycogenic (97), as were also *d*-glutamic, *dl*-aspartic, *l*-aspartic, and *dl*-pyroglutamic acids (98). The isomers of leucine were studied, revealing that *dl*-norleucine gives rise to appreciable amounts of glycogen, *dl*-isoleucine does so to a lesser extent, while *dl*-leucine, instead of causing an increase in glycogen, contributes to acetone-substance formation (99).

Using liver slices, Bach & Holmes (100) demonstrated that glycogenesis from amino acids, but not from lactic or pyruvic acids, is inhibited by insulin. From experiments on the intact animal Cherry & Crandall (101) have concluded that glucose is a better glycogen former than is lactic acid. They discovered that after the oral administration of glucose, the amount of lactic acid removed by the liver decreases and, in most instances, this organ adds lactic acid to the blood stream. The liver, however, usually absorbs lactic acid from the blood, but in hepatic disease the lactic acid tolerance curve is prolonged (102). It may be added that Shorr, Malam & Richardson (103) find that the diabetic heart, in contrast with diabetic skeletal muscle, retains its ability to resynthesize lactic acid to glycogen. An interesting observation has been made by Graham (105) that blood lactic acid is utilized for the formation of lactose by the lactating mammary gland of goats.

Glycogenolysis.—Kerr and coworkers (106) observed a diminution of cerebral glycogen during insulin hypoglycemia when the values of liver glycogen were the same as those of normal fasting animals. These authors have also reported that the glycogen, as well as the free sugar of the brain, are precursors of the lactic acid which appears during autolysis. However, neither narcotic nor convulsant drugs affect the concentration of the glycogen, free sugar, or lactic acid of the brain (107). Moreover, adrenaline, which causes glycogenolysis in liver and muscle, does not alter the concentration of cerebral glycogen (108).

Glycogen storage.—The storage of glycogen was examined by Kaplan & Chaikoff (109). They found that the water content of the liver appeared to be directly proportional to an analytical fraction, the chief constituent of which was protein, and not to the glycogen or the fat deposited in the liver. Tsai (110) has been able to differentiate two fractions of glycogen from liver, muscle, and other sources: free glycogen, and another fraction combined with protein. The latter may be in equilibrium with free glycogen, but is more stable, not being greatly influenced either by fasting or by high glucose feeding.

Deuel *et al.* (112) studied the effect of age and sex on liver glycogen, and observed that the female rat, during her reproductive life, stores less glycogen than does the male rat. Some recent evidence, however, indicates that this sexual difference, similar both in rats and in humans, may be reversed in cats (113).

THE EFFECTS OF THE ADMINISTRATION OF VARIOUS MONOSACCHARIDES ON METABOLISM

Hexoses.—Wierzuchowski (114, 115) infused glucose in dogs during six hours at a constant rate of from one to nine gm. per kg. per hr., and after careful analysis concluded that the specific dynamic action of glucose is associated chiefly with the storage reactions (glycogen, phosphoric esters, and other intermediary reactions). The chief increase of glucose oxidation occurred at a rate of one gm. per kg. per hr., at which time two-fifths of the glucose assimilated was oxidized, and three-fifths was transformed in another manner. The amount of carbohydrate oxidized increased with faster rates of infusion, and the limit of the capacity to oxidize glucose was reached at a level of blood sugar amounting to about 1000 mg. per cent. Above this level a condition is obtained in a normal dog which approaches diabetes. From a special preparation, eviscerated and functionally nephrectomized, Soskin & Levine (116) have concluded that the utilization of dextrose can occur at a normal rate in the absence of the pancreas. The carbohydrate balance was calculated from determinations of blood sugar, lactic acid, and muscle glycogen. Tissue sugar was not included in the balance.

Hexoses other than glucose have also been studied. The rise of the respiratory quotient after galactose is attributed in part by Carpenter (117) to the formation of acid products in its intermediary metabolism, as indicated by a fall in alveolar carbon dioxide. Bachmann & Haldi (118) concluded, as a result of experiments on human subjects, that a portion of ingested fructose is converted to fat. These observers obtained respiratory quotients above unity after corrections for the non-metabolic carbon dioxide liberated by the intermediary lactic acid. According to Haldi & Bachmann (119), glucose and fructose fed immediately before exercise do not increase the total carbohydrate combustion during exercise, but when the sugars are administered thirty minutes before exercise, the amount of carbohydrate oxidized is greater, indicating increased availability after the absorption of these sugars (120).

Cuthbertson, McCutcheon & Munro (121) have demonstrated that the addition of carbohydrate and fat to an adequate diet increases the retention of protein, as indicated by positive nitrogen and sulfur balances. Larson & Chaikoff (122) have shown that carbohydrate must be fed either four hours before or after the ingestion of protein

in order to have this sparing action. The effects of carbohydrate on fat and protein metabolism during diabetes are still controversial. Barker, Chambers & Dann (123) observed no nitrogen-sparing action or decrease in ketonuria in depancreatized dogs upon the ingestion of 16 to 50 gm. of glucose. On the other hand, Mirsky and coworkers (124, 125) noted that the injection of glucose in amounts large enough to insure the deposition of liver glycogen in phlorhizinized and depancreatized dogs did prevent the formation of acetone substances, and concluded that the effect is not ketolytic, but rather antiketogenic. Similarly, such huge injections of glucose exert a nitrogen-sparing action. Barker & Sweet (126), using the same preparation as Mirsky and associates, confirmed their work, and proved that under these conditions the effects of glucose on fat and protein metabolism were not due to the oxidation of carbohydrate. This new conception of antiketogenesis is suggestive and will undoubtedly be subjected further to critical study. As a result of experiments on normal fasting humans, Markees (127) agrees with the view more generally held that dextrose diminishes the concentration of acetone substances, not by preventing their formation, but by accelerating their utilization. Barnes & Drury (128) have demonstrated the absorption of ketone substances by tissues, a fact which may account for an important part of the metabolism of individuals with ketosis. It follows that this mechanism of fat utilization could also operate when the body is oxidizing fat in the absence of ketonuria. Lichtman (129) showed that the ingestion of 50 to 70 gm. of glucose caused a fall in the total fatty acids in the blood of normal dogs. This action on fat is absent in the depancreatized and the Houssay dog.

Alcohol.—The effect of fructose, glucose, and galactose on the rate of oxidation of alcohol has been studied by Carpenter & Lee (130), who noted that all three sugars increase the rate of oxidation, fructose having the greatest effect, and galactose the least.

VITAMINS

Dietary deficiencies produced by the absence of various components of the vitamin-B complex are associated with profound changes in carbohydrate metabolism. In the absence of thiamine (B_1) carbohydrate ceases to be transformed to fat (133, 134). Drill (135) observed that liver glycogen is no longer maintained in thyroxin-injected rats unless increased amounts of thiamine and of riboflavin (one of the constituents of G or B_2) are administered. No differentiation was

made between riboflavin and thiamine in these experiments. According to Martin (136), the usual responses to insulin are lost in depancreatized dogs maintained on a diet free of the vitamin-B complex. The combined effects of thiamine and riboflavin, but not those of thiamine alone, are necessary for the restoration of the action of insulin in these animals. It is, therefore, important to examine the mechanisms by which these vitamins influence carbohydrate oxidations.

Thiamine, according to Peters (137), catalyzes the decarboxylation of pyruvic acid. In support of Peters' conception, the anaërobic (138) and aërobic (139) formation of carbon dioxide from pyruvic acid have been demonstrated with the aid of thiamine pyrophosphate, the cocarboxylase of Lohmann (140). The anoxidative mechanism involves the production of acetaldehyde, while the oxidative one, that of acetic acid. Probably the oxidative process operates in animal tissues, for the presence of thiamine increases the oxygen uptake of brain slices of B-avitaminotic pigeons. The pyruvic acid which is not oxidized during avitaminosis-B exerts a secondary effect, preventing the action of lactic acid dehydrogenase (141), thus causing an accumulation of lactic acid. This explains the observations of Galvao & Pereira (142) that in rats suffering from typical symptoms of avitaminosis-B, lactate is incapable of increasing significantly the respiration of the anterior third of the cerebral cortex, although this action is retained on the brain stem.

Riboflavin, which with phosphoric acid and a protein carrier forms the yellow ferment of Warburg, is involved in the absorption of sugars from the intestine. Verzár and his coworkers (143, 144) report that the rapid absorption of glucose and galactose, in contrast with that of the pentoses, is dependent upon the phosphorylation of these two hexoses, a process for which the yellow ferment is necessary. According to these workers, after extirpation of the adrenal gland the selective action of the intestine for glucose and galactose ceases, due to failure of the synthesis of flavin phosphate (145). Deuel and coworkers (146), however, observed normal rates of absorption of glucose in rats adrenalectomized twelve to twenty days and maintained with adequate amounts of salt. In view of the various effects of the components of the vitamin-B complex on carbohydrate metabolism, it is appropriate to conclude this section with the experiments of Chaikoff & Kaplan (147). Their depancreatized dogs survived as long as four or five years when maintained on a high calorie, high protein, high vitamin diet. These workers suggest that previous

observations in which it was impossible to obtain lengthy survival were due to inadequate absorption of the diet fed.

MECHANISMS OF CARBOHYDRATE OXIDATION

Evidence for the oxidation of carbohydrate in the absence of insulin has been reviewed in the first section of this chapter. It is, therefore, of interest to examine a mechanism of carbohydrate oxidation which does not involve the mediation of insulin. Szent-Györgyi *et al.* (148) found that succinic, fumaric, oxalacetic and other four-carbon dicarboxylic acids act catalytically as hydrogen acceptors to promote the oxidation of carbohydrate in muscle tissue. Stare & Baumann (149) confirmed and extended this work. Koranyi & Szent-Györgyi (150) suggest that diabetes is caused by failure of this catalytic system in which a split product of carbohydrate metabolism, namely, pyruvic acid, is converted to a ketone substance instead of being oxidized to carbon dioxide and water. Krebs & Johnson (151) strengthen this point of view by *in vitro* anaerobic experiments, disclosing that muscle tissue can form acetone substances from pyruvic acid. These authors (152) report that citric acid, like succinic, may stimulate carbohydrate oxidation in muscle. They believe that this occurs as a result of an equilibrium between citric, α -ketoglutaric, and succinic acids, and other four-carbon dicarboxylic acids. Krebs (153), furthermore, has demonstrated the synthesis of citric acid from oxalacetic acid, and a second substance derived from carbohydrate, probably pyruvic acid. By *in vivo* experiments, Orten & Smith (154) have shown that malonic, succinic, fumaric, and malic acids are precursors of citric acid. Finally, Krebs (153) makes the suggestion that citric acid might be given to diabetic patients in order to combat ketosis. This conception appears to be based on sound *in vitro* experiments, except that the catalytic effect of these C_4 -dicarboxylic acids has not been studied on diabetic tissues. It is difficult, moreover, to reconcile the observations made on excised tissues with those on the intact animal, in which Deuel, Hallman & Murray (155) find that succinic acid is ineffective in preventing the ketonuria of rats fasted and then fed a high fat diet. Although Koranyi & Szent-Györgyi (150) report improvement following the administration of succinic acid to five patients with diabetes, Lawrence, McCance & Archer (156) and Dunlop & Arnott (157) have been unable to confirm these observations.

Schoen & Gerard (158), using brain extracts, note that lactate

can be oxidized by cerebral enzymes without the intermediation of the dicarboxylic acids. Jowett & Quastel (159) have shown that hydroxymalonate inhibits lactate oxidation by the brain to a much greater degree than glucose. They conclude that glucose oxidation does not necessarily proceed through lactic acid. Despite the progress made in the study of carbohydrate oxidations, a complete conception of these mechanisms and of their antiketogenic effects still awaits development.

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FAT METABOLISM*

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General role of fats in metabolism.—The general role and importance of fat in animal and human diets has been discussed during the last year in a series of studies. Bull (1) has written a *Biochemistry of Lipids* which contains valuable biological sections. Anderson & Williams (2) have produced a remarkable review of the role of fat in the diet, especially fat as a carrier of vitamins, its sparing action for vitamin B₁, and the relation of dietary fat to body and milk fat, for which the survey is complete. An extensive experimental study of Benedict & Lee (3) also contains many references to the literature on lipogenesis in the animal body, and many on the technique of animal calorimetry. The experiments were done mostly on the goose, and the study contains many details of the general physiology of this animal.

In their experiments the relation between oxygen consumption and heat production (directly measured), in the process of transformation of carbohydrate into fat, was determined. At an R.Q. between 1.17 and 1.39, 5.002 kilocal. per liter of oxygen were produced. This oxygen-energy relationship was not different from that obtaining during the combustion of pure carbohydrate.

Until now it has been generally supposed that fat tissue has no important role in the metabolism of the whole body. But the experiments of Benedict & Lee are to the contrary. If geese were artificially fattened, their body weight increased enormously through the deposition of fat. Adult geese increased from 2.5 to 7.5 kg. while their protein content remained constant. But their heat production increased in proportion to the body weight, from 150 kilocal. to 400 kilocal. in 24 hours. If calculated in terms of unit weight, then for animals of between 4.5 and 7.5 kg., the basal metabolism was constant—about 54 kilocal. per kg. per day. While it is known that the metabolism in animals of different body weight is different per unit of body weight, here this is not true. Thus one has to conclude that the fat tissue plays a considerable part in the heat production of these animals.

The same was also shown in mice. The heat productions of

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an albino mouse (21 gm.), a fat mouse (50 gm.), and a dwarf mouse (8 gm.) were compared. Calculated per unit body weight they produced 135, 110, and 111 kilocal. per kg. per day. The fat mouse produced, per gram of body nitrogen, twice as much heat as the albino mouse. Both had almost the same nitrogen content, but one had 2.54 gm., the other 31.5 gm. of body fat. Thus, in these animals also the fat tissue must have taken part in heat production.

The proportion in which fat is burned during fasting seems to be different in different animals. Synephias (4) found that the relation in the pig is 6:94, mouse 8:92, guinea pig 9:91, rat and dog 10:90, man 15-20:75-80, rabbit 30:70. Respiration experiments are also published by Lecoq & Joly (5) in this connection.

It was thought by Forsgren (148) that a periodicity could be seen in the function of different organs, and this was the basis for a study by Holmgren (6) on fat metabolism: Rats were fed a diet rich in fats; by means of chemical and histological methods he found variations in the fat content of the intestinal mucosa, with a maximum at night; he believes that this is the result of an endogenous rhythm in the absorption capacity of the epithelium. Such a rhythm could also be seen in the zymogen granules of the cells of the pancreas and in the fat content of the lungs, with a maximum between 6 p.m. and midnight. Liver fat, and also liver glycogen, did not show such a clear periodicity, but there was nevertheless a maximum at night.

A special adaptation to extraordinarily high fat diets is observed in East-Canadian Eskimos, 27 individuals of whom were studied by Corcoran & Rabinowitch (7). In contrast to Europeans, they have no ketosis on these diets. Their blood-fat content is also not high in any of the several fractions. It was concluded that they must have a very active, but special, mechanism of fat oxidation.

From the work of recent years, it is now believed, especially from the experiments of Burr, that the rat is unable to subsist on a diet free from fat, and that the mixed acids of linseed oil are indispensable. Moore (8) has subjected the liquid and solid acids of linseed oil to a brief saponification or to a long treatment with potassium hydroxide. Changes in the absorption spectra of these and other fats were induced by such treatment, showing an alteration of the molecule, but their biological activities in such different spectroscopic states were not different. As he remarks,

however, it is not impossible that small amounts of more highly unsaturated acids might have been present.

The nutritive value and digestion of different fats.—This has been studied by a series of Italian authors. Baglioni & Famiani (9) showed that hydrogenated olive oil and butter fat maintain the growth and weight of rats just as well as the non-hydrogenated products. They conclude that unsaturation is certainly not important for the better utilization of fat. Also Peretti & Reale (10) have stated that there is no difference in the absorption of fats of different degrees of unsaturation. Ducceschi & Roncato (11) found in men that from five differently purified olive oils 93.4 to 95.8 per cent was absorbed when about 100 gm. were ingested. There was no difference between oils which were differently purified or rectified.

Steenbock and his coworkers (12) have also systematically studied the absorption of different fats from the rat intestine, and observed the action thereon of many different substances. Hydro-tropic substances (other than bile salts) did not increase absorption, and in greater concentrations, as with other substances, had rather an inhibitory influence. In pathological conditions—avitaminoses, anemia, etc.—there was always a decreased absorption.

Of all the fats studied, castor oil was the only one which was poorly absorbed. Valette & Salvanet (13, 14, 15) have searched for an explanation of this. They state that castor-oil fat is well split by lipase and dissolved by bile, but the absorption is inhibited since the split product, ricinoleic acid, has a laxative action. This might be explained thus: at pH 7 sodium ricinoleate is but slightly hydrolyzed; it has therefore a solvent action on the lecithin and thereby demolishes the cell membranes.

The possible influence of fats on the production of gastric juice was studied by Fierens & De Nayer (16), who produced a gastric secretion by giving histamine. If arachis oil were given at the same time, they found an inhibition of gastric juice production. This they explained as an action on the production of a hormone "Chalon," which, as they state again, is not identical with secretin or cholecystokinin.

It has often been shown that fat absorption influences calcium absorption in a positive way, and it is thus somewhat unexpected to find that the results published by Nakamura (17) are to the contrary. When milk was fed and calcium lactate was given, cal-

cium soaps were formed, but these were only partly soluble in bile. In dogs, after feeding calcium lactate, the quantity of calcium soaps in the faeces even increased, but this increase was very slight in his experiments.

Composition of different fats.—For methods of estimation of fats, we would refer especially to papers by Boyd (18), Rappaport & Wachstein (19, 20) and Ackermann (21). Not only for practical purposes, but also from a theoretical point of view, it is interesting to note that during the storage of blood plasma there is a decrease in the phospholipid content in one to three months and later in the cholesterol-ester content also; meanwhile "neutral fat" increases but no appreciable change in total fatty acids is found (18). It makes no difference whether the plasma is kept at room temperature or in a refrigerator, with or without light, or even in alcohol or alcohol-ether.

Much work has been done on the constitution of different fats. Anderson, Reeves & Stodola (22) describe the lipids of the tubercle and lepra bacilli. Wagner-Jauregg (23) has isolated from tubercle bacilli, destroyed by heat, various fatty acids, especially $C_{29}H_{58}O_2$ (tuberculinic acid). Bernhauer & Posselt (24) found unsaturated fatty acids in *Aspergillus niger*, especially lignoceric acid, which is known from other mould- and yeast-fats.

The depot fats of different fresh water and marine fishes were analyzed by Lovern (25). Their fatty acids (C_{16} to C_{20}) form two distinct types. In marine fishes there are relatively more of the higher, in fresh water fishes more of the lower, unsaturated fatty acids. Hilditch & Paul (26) found the abdominal fat of a very young Ceylon lizard (*Varanus salvator* Laur.) to be intermediate in character between those of land and marine animals, as is true of other amphibians and reptiles. The fat is almost completely liquid at 20° C. and contains 4 per cent myristic, 29 per cent palmitic, 10 per cent stearic, 12 per cent C_{16} unsaturated, 40 per cent C_{18} unsaturated and 5 per cent C_{20} unsaturated fatty acids.

Casein fat, i.e., the lipid matter of precipitated casein, is 1 to 2.5 per cent of the milk fat. An analysis by Stevenson & Bacharach (27) shows that only the absence of phospholipins distinguishes it from butter fat, with which it is identical in all other respects.

Extensive studies of ox-depot fats were published by Hilditch & Longenecker (28), who also give a good survey of the earlier

literature. Their analysis indicates certain general features of land-animal depot fat, which contains 38.0 to 40.4 per cent oleic, 26.5 to 31.0 per cent palmitic and 20.1 to 25.4 per cent stearic acid. There are two unsaturated members of the oleic acid series (myristoleic and palmitoleic acid), which have not previously been reported as components of the fat stores of higher land animals. The C_{18} unsaturated acids consist chiefly of oleic acid and some linoleic acid. Small amounts of myristic, arachidic, arachidonic and possibly lauric acid may be present. Hilditch & Shorland (29) describe the liver phosphatides as "characterized by the presence of increased proportions of stearic, C_{20} and C_{22} unsaturated acids together with diminished proportions of hexadecenoic acid, as compared with the corresponding liver 'glycerides,'" and "a marked tendency of liver phosphatides as compared with liver glycerides to contain acids of higher mol. wt." Linoleic acid is not present in the fatty acids of the cow and ox liver. The liver lipids of dogs, fed with different fats, show no differences except in the iodine numbers of the total fatty acids (30).

Koppenhoefer (31) has called attention to the fact that the different lipids of the skin are localized in different histological layers: in the corium, phospholipids and sterols are constituents of the protoplasm; triglycerides appear near the subcutaneous region; in the epidermal region are waxes, together with phospholipids and sterols, produced in the "basal" epidermal division as a "sebum."

The lipid content of rat brain and liver at different ages is described by Lang (32).

Wendt (33) reports that extracts of the brown coloured fat tissue of the hibernating hedgehog produced apathy in rats and decreased their basal metabolism and pulse rate. This might indicate the tissue to be endocrine rather than depot fat.

We cannot refer here to the chemistry of fats, but attention must be called to important steps in their synthesis by Hilditch & Rigg (34) who published descriptions of the synthetic production of monoglycerides of palmitic, stearic and oleic acids, and especially by Fischer & Baer (35) who succeeded in synthesizing the triglyceride α -stearyl- α' - β -dipalmityl glycerin (optically inactive.) Starting from α -acetone glycerin they also synthesized glycerin- α -phosphoric acid, which is present in lecithin and is also produced in carbohydrate metabolism. They suggest that this substance, via the phosphatides, might be a stage in formation of the optically active glycerides of fat.

Absorption of fat from the intestine.—Our views on the mechanism of absorption of fat from the intestine have changed greatly during recent years. A complete bibliography and discussion of the subject can be found in Verzár's (36) *Absorption from the Intestine* (pp. 150 to 211, 286). The purely physical mechanism by which fatty acids are taken up by the mucosal epithelial cells has been elucidated in a very interesting paper by Rossi (37) who has called attention to experiments which he had already published in 1908 and which he is now able to explain. Verzár & Jeker (38) and Jeker (39) have described how fatty acids can first be shown in the mucosal epithelial cells and then later how they are synthesized to neutral fat. Fatty acids are dissolved in bile acids and diffuse in such a solution into the epithelial cell. Rossi shows that even in an intestine which is fixed with formaldehyde, the same histological picture can be seen on immersion in a solution of fatty acids with bile acids. After some time the fatty acids can be shown as fine granules inside the cells. This is not the case if one puts the intestine in an emulsion of neutral fat in bile acids. Fatty acids alone, without bile acids, also diffuse, but much more slowly. This is in complete agreement with the observations on living animals. Rossi then found that if he put the formalin-fixed intestine into a fat solvent, it lost the capacity to take up the fatty acids. Thus the entrance of fatty acids into the cell is a purely physical diffusion process requiring certain lipid structures (possibly the Golgi apparatus).

The fact that some Japanese authors (40, 41) have found that neutral fat, finely emulsified by passage through a "homogenisator," can be absorbed from the isolated ileum and the large intestine without being split to fatty acids by lipase, is no contradiction of the generally accepted view of fat absorption. Normal fat absorption occurs in the duodenum and the jejunum, always after splitting of the fat by lipase. From their own experiments on dogs, it is clear that from the small intestine 66 to 73 per cent was absorbed in 3 hours while from the large intestine 65 to 83 per cent was absorbed without splitting only in 24 to 48 hours. From emulsions which were even as fine as milk, nothing was absorbed! Thus it seems that the epithelial cells are not impermeable for such extremely fine emulsions, especially if they are under pressure as in a tied intestinal loop, but the physiological way of absorption, i.e., after lipolysis, is much more rapid.

Nor is Breusch's paper (42) a contradiction of the present view that fats are absorbed after splitting and solvation of the fatty acids in bile acids. This author found no diffusion through parchment membranes of bile acid solutions of saturated fatty acids from palmitic acid (C_{16}) or oleic acid upwards. Saturated fatty acids from C_{14} (myristic acid) and unsaturated from linolic acid downwards dialysed well in bile-acid solution. Since several authors (43 to 46) using different methods of diffusion and fatty acid estimation had observed, to the contrary, the diffusion of the higher fatty acids also, these results are to be explained by a low permeability of Breusch's membranes, which allowed only the lower fatty acids to pass. Since such experiments on membranes always remain as models, nothing can be concluded from his negative results as to the mode of fat absorption in the body.

Holwerda (47) has adopted a clearer and more precise method for studying the mechanism of solution of fatty acids by bile acids. Sodium glycocholate dissolves caprylic acid and its higher homologues and the "association products" are in many ways the same as those obtained by Verzár & Kuthy (149) from higher fatty acids.

Lymph fat.—Artom & Peretti (48) found the lipid content of the thoracic duct lymph in dogs during fat absorption to be 2 to 5.4 per cent, and during fasting 0.25 per cent. Of this 88 to 96 per cent were triglycerides, and only 2 per cent were free fatty acids and soaps. The molecular weights of the lymph fatty acids were lower than those of the fat which was fed and the iodine number was higher. They explain this by assuming that phosphatides are produced in the intestinal mucosa during absorption.

Reiser (49) studied human thoracic duct lymph from a lymph fistula in a patient on a low fat diet. This lymph contained about 300 mg. per cent neutral fat, about 70 mg. per cent phospholipid, and 30 per cent cholesterol of which about 20 per cent was free. The fatty acids had a mean molecular weight which was close to that of stearic or oleic acid.

The lymph of rabbits (taken from a peripheral lymph vessel) contains 2.5 mg. per cent of ketone bodies (50).

Blood lipids.—Frazer & Stewart (51, 52) after a short bibliographic study of the ultramicroscopic particles of blood (the so-called haemoconia or chylomicrons), give a good description of the picture seen in blood plasma with dark-ground illumination. There is a definite change after a single meal, the number of the "dull," "small,"

and "large bright particles" increasing about fivefold, with a maximum after two to three hours. Chemical estimations give curves which coincide with simultaneous particle curves, showing that the particles are certainly neutral fat, while the rise in blood cholesterol occurs after the particle curve has started to decline. Hayami (53) states that in cases of lipaemia he found lipid granules even inside the erythrocytes and leucocytes.

The blood lipids have been estimated with chemical methods in many cases. Effkemann (54) gives the following mean values for foetal blood and serum.

LIPID CONTENT OF FOETAL BLOOD AND SERUM

	Fatty Acids	Free Cholesterol mg. per 100 cc.	Cholesterol Esters	Leci- thin
Venous blood	105.7	78.2	17.1	224.8
Arterial blood	104.8	75.9	14.5	214.8
Venous serum	127.5	22.0	49.6	121.1
Arterial serum	126.8	21.2	44.4	120.0

Of the various factors which influence the quantity of blood lipids, Hansen & Wilson (55) studied serum lipids in relation to intermediary fat metabolism in hunger and on diets poor and rich in fat. The lipid content varies with the intensity of the fat metabolism. But, as Corcoran & Rabinowitch (7) had shown in Eskimos accustomed to very high fat diets, the blood lipids are no higher than in Europeans on low fat diets. On a very low protein diet, such as would ultimately cause edema, the quantity and quality of the serum lipids of dogs does not change (56).

Blood lipids are markedly influenced by disturbances of internal secretion. Boyd (57) has studied the relation of the thyroid to blood lipids. Complete thyroidectomy in man produces an increase in all the plasma-lipid values, but does not change the lipid content of the red blood cells. In hyperthyroidism (58) the opposite is true, namely, lipopenia occurs. There were also changes corresponding to an inverse relationship in cases under treatment (59), i.e., if the function of the thyroid increased, the plasma lipids decreased.

Even more pronounced are the changes of the blood lipids under the influence of the pancreas. In normal man, after one to two days of a carbohydrate-poor diet, the blood lipids (total fatty acids, cholesterol, and especially cholesterol esters) increase. Insulin does not influence this lipaemia which, however, disappears when carbohydrate is again given.

In diabetics it is impossible to decrease the lipaemia with carbohydrates, but this can easily be done with carbohydrates together with insulin (70). In Eck-fistula dogs no striking differences were seen in blood lipids compared with normal animals (60).

To determine the form in which fat is transported, former authors have used iodized fat. Aylward, Blackwood & Smith (61) found that if this was fed to cows, its concentration in the blood became maximal after one and one-half to two days and fell within five days. The amount secreted in the milk was directly related to the amount of neutral fat in the blood. Thus they returned to the conclusion that fat is transported in the blood chiefly as neutral fat and not as phosphatides, nor are the latter the precursors of milk fat.

The intermediary production of phospholipids.—It was believed during recent years that the synthesis of fatty acids to neutral fats during fat absorption in the intestinal mucosa took place through the intermediate production of phospholipids. This problem has now been studied by "labeling" a molecule through the use of an isotope of one of its atoms. The radioactive isotope of phosphorus (^{32}P) was first used in biological problems by Hevesy (62). Then Artom and coworkers (63) injected subcutaneously, or gave *per os* (64) sodium phosphate with ^{32}P . They then extracted the phospholipids of different organs and found the ^{32}P mostly in the intestinal mucosa, the liver, and the kidney, practically none being found in muscle and the central nervous system. Hahn & Hevesy (65), however, using the same methods, found "labeled" phosphorus in the lecithin of the brain of rats, as early as one hour after injection. This was taken to be a proof of constant breakdown and rebuilding of lecithin in the brain tissue.

Artom explains his results in the same way as does Sinclair, as a phosphatide formation in the intestinal mucosa, and especially as a direct participation of the phospholipids in fat metabolism. But this was again contradicted by Hevesy & Lundsgaard (66) who also fed radioactive sodium phosphate together with fat. During fat absorption there is a lecithinaemia, but since this lecithin does not contain the radioactive phosphorus, it cannot be produced from the absorbed fatty acids and the radioactive phosphate. They believe that it is "mobilised" phosphatide and that "during fat absorption lecithin is formed outside the intestinal tract." This conclusion seems, however, not to be finally proved. Phosphatides might be formed during fat absorption in the intestinal mucosa, but not neces-

sarily from the radioactive ^{32}P which is present in the intestinal contents. It has never been supposed that the intermediary phosphate production during fat absorption requires absorbed phosphates.

Ackermann (67) has also studied the role of phosphatides in fat absorption. She has tested the absorption of lecithin from the frog intestine and confirmed the fact that it is split before absorption. From its fatty acids, neutral fat is synthesized. The quantity of phosphatides does not change in the intestinal mucosa during fat absorption. This is also in agreement with Sinclair's work which was, unfortunately, misunderstood.

By the same method as in former experiments, Sinclair has now shown with McConnell (68) that elaidic acid, when introduced into the body, finally reaches the brain and is built into the lecithins and cephalins. More, however, goes to the liver- and muscle-phosphatides. It also (69) passes through the rat placenta and mammary glands.

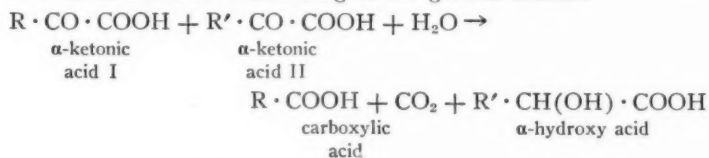
Intermediary fat metabolism. Oxidation of fatty acids.—When fat is absorbed, it may either be oxidized immediately or deposited in the fat depots, i.e., the fat tissue. Both alternatives seem to be possible. In animals which have been previously fed with carbohydrate or which are fasting, high fat diets produce ketosis. This is generally thought to be the sign of an intensive oxidation of fats. It is also known, however, that the same high fat diets do not produce ketosis if the diet constantly contains liberal quantities of fat. Thus, intensive oxidation of fats and ketosis seem to occur after fat feeding if there is a need of fat; but this does not mean that some depot fat is not deposited at the same time, and this portion may vary greatly under different conditions. Schoenheimer, Rittenberg, and coworkers (71), from experiments in which deuterium-labeled fat was used, show that under their experimental conditions the food fat is never oxidized immediately but is first deposited in the fat tissues (72).¹ However, synthesis and destruction of higher fatty acids proceeds constantly in the body (73). If mice are maintained on a carbohydrate diet and their body fluids are kept constantly saturated for a long time with D_2O administered by injection and *per os*, deuterium will be found eventually in their saturated and unsat-

¹ This is true for C_{16} and C_{18} fatty acids, but not for butyric and caproic acids, which are not found in the fat tissue after feeding, but are "rapidly and completely disposed of."

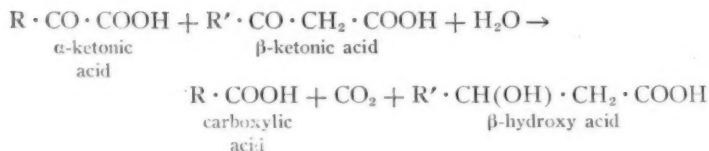
urated fatty acids and also in their cholesterol (cf. p. 184). The deuterium content of the fatty acids reaches a constant value from which a "half-life time" of about five to nine days was calculated. Stearic and palmitic acids did not differ in their deuterium content; unsaturated fatty acids contained less. For cholesterol the deuterium content rose more slowly, so that a "half-life time" of about fifteen to twenty-five days was calculated. The different fatty acids are transformed into each other (74), so that if mice are fed for five days with deuterostearic acid, deuteropalmitic acid can be isolated from the body fat; saturation and desaturation of fatty acids also goes on constantly (75). The authors realize that there are some difficulties in interpreting their results, especially when the action of desaturating enzymes is taken into account. They have shown that concentrated D_2SO_4 exchanges deuterium with palmitic acid (76), and there might be some doubt as to whether, under the conditions in the body, such an exchange might not be possible also by enzymatic actions, which would then influence the explanation of the experimental results.

Schoenheimer (77) has published an account of his technique and experimental results in his Harvey Lecture. While his method of studying fat metabolism certainly brings several interesting facts to light, one has to be careful with interpretation, as Bonhoeffer (78) has rightly remarked in the Bunsen Society's deuterium discussion. If there is a positive finding for deuterium, the experiments are decisive. But there is always the possibility that the fats, through some unknown process in the body, lose their deuterium even if bound to carbon. In such cases the history of the substance cannot be followed. The exchange generally takes place with water and "also the opposite may happen, that such compounds take up deuterium in a non-exchangeable form. Such cases have been found more than once." (Cf. pp. 178, 184.)

It seems to be extremely difficult at present to reach any final conception of the role of ketone bodies in intermediary fat metabolism. This is clear from the paper of Krebs & Johnson (79) who explain, from experiments on tissue slices, that ketonic acids can react in animal tissues according to the general scheme.



or



Examples are given in which α -ketonic acid I, as well as α -ketonic acid II, are represented by pyruvic acid. In other cases the α -ketonic acid is pyruvic acid or α -ketoglutaric acid and the β -ketonic acid is acetoacetic or oxalacetic acid. Both reactions are mechanisms by which α -ketonic acids are broken down in the animal body. The oxidation is not brought about by molecular oxygen, but by a dismutation, i.e., by an intermolecular oxido-reduction. The oxidizing agent for the ketonic acid is a second molecule of ketonic acid which is reduced to the corresponding hydroxy acid. Both reactions appear to play a part in the normal oxidative breakdown of carbohydrates, of fats, and of the carbon skeleton of amino acids.

Verkade's work on the oxidation of fatty acids is well known; together with his coworkers he showed that in dog and man, after feeding different monoacid saturated triglycerides, dicarboxylic acids appeared in the urine ("diaciduria"). This must be explained as an oxidation of the methyl group, i.e., ω -oxidation of the fatty acid components. The degree of ω -oxidation is rather variable. Flaschen-träger & Bernhard (80) have emphasized that it is obtainable only with the fatty acids C_8 to C_{11} . But Verkade and coworkers (81) now show that all saturated fatty acids are oxidized by ω -oxidation. They call attention to the fact that it is not at all a rare form of breakdown. However, triheptylin and triundecylin do not produce diaciduria; tricaprolylin is only slightly diaciduric, and trinonylin and tricaprins are practically void of effect. Kuhn, Köhler & Köhler (82) show further that the polyene carboxylic acids $[\text{H}_3\text{C}-(\text{CH}=\text{CH})_n-\text{COOH}]$ are also subject to ω -oxidation so that ω,ω -dicarboxylic acids occur. Thus, through feeding sorbic acid they obtained *trans*, *trans*-muconic acid.

The next step for all normal saturated dicarboxylic acids is, according to Verkade, that they are further decomposed through a biterminal β -oxidation. Artom (83) explains that dibasic fatty acids are not only broken down through two consecutive β -oxidations, but also through simultaneous β -oxidation at both ends of the molecules

(β , β -oxidation). He quotes, especially, experiments of Mazza (150) in which γ -ketones could be shown among the oxidation products of surviving liver slices. The fact that diacetyl is often found in the metabolic products of different organs may be explained in this way.

According to Bernhard & Andreae (84) after feeding the C_8 and C_{10} dicarboxylic acids (suberic and sebacic) and sometimes also adipic acid to men and dogs, they were largely excreted; they are used much less completely than monocarboxylic acids. Succinic acid, on the contrary, is completely oxidized. This supports the view of Flaschenträger & Bernhard (80), already mentioned, that the main quantity of fat used in metabolism does not undergo ω -oxidation, since dicarboxylic acids are only partly β -oxidized and to a greater extent are excreted unused.

Ketosis.—The alimentary production of ketone bodies has been studied by many authors. Brentano & Markees (85) after feeding great quantities of C_4 to C_{18} fatty acids always found an increased ketonaemia, but none was seen after feeding C_{2n-1} and unsaturated fatty acids. Normally the ketone bodies are quickly used up; in fact the ketonaemia could be shown only under special circumstances such as after adrenaline or in creatinuria. These considerations seem to reduce the significance of their result. Deuel and coworkers (86) fed to fasting male rats the ethyl esters of many fatty acids. Those of C_{2n-1} fatty acids, such as propionic, valeric, heptonic, pelargonic, and undecylic acid produced no ketonaemia, while those of acetoacetic, butyric, and caproic did, and even more so those of caprylic, capric, lauric, and myristic, and also of palmitic, stearic, and oleic acids. They then postulated that the last three break down into at least three fragments per molecule, each of which is capable of forming acetone bodies.

Caproic, butyric, and C_{2n-1} fatty acids break down by β -oxidation. C_{2n} fatty acids (C_8 to C_{14}) are broken down by γ - and ζ -oxidation ("multiple alternate oxidation") (87).

Friedemann (88) injected sodium acetoacetate into dogs intravenously, and by a series of elaborate experiments came to the conclusion that acetoacetate is metabolized in these animals by direct oxidation. Califano (89) studied, by means of the Warburg method, the oxidation of different fatty acids in normal livers and in fatty livers after phosphorus poisoning. In the latter case dehydrogenation was somewhat decreased. Leites & Odino (90) showed a marked ketogenesis in the autolyzing liver of starved animals; a

certain amount of glycogen, however, was necessary. Phosphorus-poisoned livers, on autolysis, produce even more ketone bodies. Further, Leites & Odino (91, 92, 93) believe that the lungs take part in fat metabolism through production or oxidation of ketone bodies. Lung tissue, on autolysis, also produced ketone bodies. Intravenous injection of butyric acid often increased the β -hydroxybutyric acid content of the lung, but the arterial blood contained more or less than the blood of the right heart. It might be mentioned here that after experimental fat embolism of the lungs, the fat disappeared within twelve hours; it may have been digested (94).

The ketone bodies which are produced on a high fat diet are utilized by the tissues, as Barnes & Drury (95, 96) showed. There are constant differences between the ketone-body content of the arterial and venous blood, that of the latter being about 1 mg. per cent lower. Choline, which prevents the accumulation of fat in the liver on high fat diets, does not increase ketonaemia or ketonuria, thus does not increase the rate of fat oxidation ["if ketonuria during fasting is an evidence of such oxidation . . ."] (Deuel *et al.*, 97).

Ketosis, on the other hand, is abolished by sugar. In different forms of ketosis, to which we shall refer later, Murlin and coworkers (98, 99) have tested different sugars. In the dog on a high fat diet galactose has the greatest and glucose the lowest ketolytic effect; fructose is intermediate. In the depancreatized dog glucose and galactose were equal and fructose was without effect. In humans (100, 101) glucose was more effective than fructose. A late sugar dose following an early one was very effective.

Fatty liver.—The accumulation of fat in the liver has been reported upon in many papers. There are many ways in which fatty livers can develop. By forced feeding of carbohydrates enormous quantities of fat can be produced in the goose (3, 102, 103). This liver fat is more saturated than normally, and more so than the depot fat. Rosenthal (104) has discussed the relationship between the morphological changes and the chemically analyzed fat content of the liver, using mainly sudan-red for the histological studies.

In continuation of previous studies on dietary fatty livers in rats, Channon, Jenkins & Smith (105) investigated the degree to which certain fats of widely varying chemical composition accumulated in the liver when fed as 40 per cent of the diet, with a choline intake of about 1.5 mg. daily. No relationship was found to exist between the amount of fat in the liver and that in the carcass. The saturated

fatty acids of both bear a close resemblance in the proportions present and in their mean molecular weight. Unsaturated acids were less closely related.

How complicated the conditions are under which fatty livers are produced can be seen from the fact that while Beeston & Channon (151) had found that in rats on a diet containing 5 per cent casein and 40 per cent lard, supplemented with 0.5 per cent cystine, the liver-lipid content was increased by 57 per cent, Tucker & Eckstein (106) added to the same diet 0.5 per cent methionine, and then found not only no increase, but even a decrease of 41 per cent of the liver fat. No explanation is offered for this result which is a contradiction of the generally accepted belief that cystine and methionine have a similar function in metabolism.

Vitamin B₁, according to McHenry (107, 108), markedly increases the amount of fat in the livers of rats (on a high fat and low choline diet), sometimes to values three to four times higher than in the livers of rats without B₁. [Salmon & Goodman (109) described an increase in body fat on adding autoclaved yeast to the diet, but it is not clear whether this is due to vitamin B₁ or to choline.] It might be mentioned that a fatty liver can be the result of many unspecific factors, as, for instance, has been described after experimental infection with *B. typhosus* (101).

As is well known, choline is effective in preventing dietary fatty livers. It was shown by Channon, Platt & Smith (111) that synthetic homocholine (trimethyl- γ -hydroxypropyl ammonium hydroxide) is even more effective. It was fed in quantities of 4.5 to 14.0 mg. per day. The triethyl- β -hydroxyethyl ammonium hydroxide was much less active than choline, and tripropyl- β -hydroxyethyl ammonium hydroxide was without action. Thus, replacing three methyl groups in choline with ethyl groups leads to a loss of activity of about one-third, and when three propyl groups are substituted the compound becomes inactive.

Dragstedt (152) had isolated a hormone from the pancreas called "lipocain." This seemed to have a "lipotropic" effect, i.e., the livers of rats on high fat diets did not become fatty. Aylward & Holt (112) failed to observe any effect of pancreas feeding other than that due to the contained choline. But Kaplan & Chaikoff (113) claim that they have demonstrated in the pancreas two factors which are active in lipid metabolism. One inhibits the production of fatty livers in depancreatized dogs kept alive with insulin. While choline, given in

daily doses of 3 gm. for a long time, also has this inhibiting action, it seems to be difficult to decide whether it is identical with the pancreatic liver factor. Choline, however, does not raise the blood lipids in depancreatized dogs as does the pancreatic "blood-factor," and to this extent the latter differs from choline.

Excretion of fat.—It is known that in a closed, isolated intestinal loop lipids are excreted. It has, however, been difficult hitherto to determine the normal proportion of fat secreted into the bowel. Shapiro, Koster, Rittenberg & Schoenheimer (114) tried to accomplish this by feeding fat "labeled" with deuterium to patients with a bile fistula which could be opened or closed. They report that when the bile was flowing to the exterior nearly all of the ingested fat was found in the excretions as fatty acid. But this was not the "labeled" fat which they had fed! Of the ingested labeled fatty acids, about 70 per cent was lacking and they believe that this was absorbed. Nearly the same quantity of unlabeled fatty acids was excreted through the intestinal mucosa! This finding presents a number of new facts, namely: (a) that fatty acids are absorbed almost quantitatively without bile; (b) that at the same time about the same quantity of fatty acids is secreted into the intestine and is not reabsorbed. It is not clear why such a secretion of fats should go on only in the absence of bile and not when bile is normally flowing into the intestine. The quantitative agreement is astonishing. Whether the authors' experimental procedure permits of these far-reaching conclusions has been discussed above (cf. p. 173).

Hormonal regulation of fat metabolism.—It was shown by Verzár & Laszt (115) that adrenalectomy abolishes the fatty livers produced by phosphorus poisoning, or fasting, in the rat. Ordinarily, adrenalectomized rats had 4.3 per cent fat in the liver. This remained so even after five days of injections of 0.5 mg. phosphorus. This was explained as an inhibition of the intermediary production of phosphatides. It was taken to be a parallel to fat absorption which is also inhibited after adrenalectomy. As in their studies on sugar absorption, here again iodoacetic acid inhibited the fat mobilization and the accumulation of liver fat just as did adrenalectomy. This supports the explanation given above, that it is a phosphorylation which is inhibited. But whether this explanation is right or not can only be finally stated if we have a complete picture as to what is really the meaning and mechanism of fatty liver production. It was then shown by these authors that when adrenal cortical hormone ("Cortine," or

"Eucortone") was injected in these adrenalectomized animals, fat again accumulated very intensively in the livers; fatty livers with 8.2 per cent fat were seen. This proves that it is the hormone of the adrenal cortex which is necessary for the production of fatty livers.

MacKay & Barnes (116, 117) have emphasized in a series of excellent papers that in all cases of ketosis fatty livers are produced. Ketosis can be produced not only by feeding high fat diets, but also by fasting, by alkalosis, by adrenaline, by pancreatectomy, by phlorrhizin, and by the injection of ketogenic anterior pituitary extracts.

As fatty liver and ketosis generally go together it was necessary to determine how adrenalectomy affects ketosis. It was shown by MacKay & Barnes that the ketosis due to anterior pituitary extracts and fasting, as well as the fatty liver, can be abolished by adrenalectomy. Adrenalectomy also abolishes the fatty liver resulting from total pancreatectomy (118). MacKay & Barnes showed also (117) that adrenal cortical extracts increase the ketosis of fasting rats. It must be mentioned, however, that Anselmino & Hoffmann (119) had found, on the contrary, that the ketonaemia produced through their anterior pituitary hormone ("*Fettstoffwechselhormon*") could be inhibited by adrenal cortical hormone, which in their experiments also inhibited the disappearance of glycogen caused by anterior pituitary extracts.

The conclusion seems to be that the ketogenic and fat-liver-producing extracts of the anterior pituitary act through a stimulation of the adrenal cortex. This was supported by Reiss, Epstein & Gothe (120) by the observation that adrenalectomized and hypophysectomized rats generally cannot deposit fat. Corticotropic anterior pituitary hormone inhibits this if the adrenal is present and gives, even in normal animals, an increased deposition of fat. The same is produced by the adrenal cortical hormone. Both hormones decrease the lipid content of the blood in man and dog. The so-called "lipoitrin effect" of anterior pituitary lobe extracts is, so they say, identical with this. While these statements could explain many pathological conditions, caution must be expressed against too wide generalizations. For instance, Chaikoff *et al.* (121) state that after hypophysectomy in dogs the blood-lipid concentration is unchanged, and if later pancreatectomy is performed fatty livers and lipaemia occur. Further, the "lipoitrin effect" (decrease of blood fats) is given by anterior and posterior pituitary extracts, and according to Strauber (122) this is the action of one and the same substance.

In normal dogs glucose causes a fall in the total fatty acids of the blood. But the lipaemia of the depancreatized dog is not diminished by the ingestion of glucose, nor is that of the hypophysectomized and depancreatized dog (123). The fat-mobilizing hormonal effect of the pancreas, which Dragstedt (152) had found and which is discussed above in its relation to choline, has been confirmed by MacKay (124) in experiments on rats. In human diabetes hyperglycaemia and hyperlipaemia seem to be abolished by insulin at least in cases which are not too severe (125). The fasting ketosis in patients with simple obesity is often greater than in the non-obese, but some obese have also a very slight ketosis. MacKay (126) suggests that in these cases there is some endocrine disturbance.

There is a difference between the sexes in regard to ketosis. In humans and rats, females show a greater tendency to ketosis. In cats, males excrete about four times as much ketone bodies as females (127). Castration brings the two values together, and testosterone strongly increases it again.

The lipoitrin effect can also be produced by stimulating the hypothalamus in the dog by an injection of ammonia into the base of the third ventricle, as van Bogaert & van Meel (128) have shown. This, of course, is in good agreement with the known facts of a hypothalamic-pituitary connection. We would explain this fact then as a stimulation of the hypothalamus \rightarrow anterior pituitary \rightarrow adrenal cortex.

Nervous regulation of fat metabolism.—The fat depots are not only influenced by hormones but also directly by the nervous system. This was long suspected but no clear proof had been produced. Beznák *et al.* (129) cut the splanchnic nerve on one side in the cat; when the animal was starved the perirenal fat decreased one to three times more on the intact than on the denervated side. If fat stained with sudan was fed, the concentration of the dye on the denervated side was about one-fourth that of the normal side. Also the unilateral extirpation of the stellate and superior cervical ganglia has an effect on the amount of pericardial fat, as has unilateral extirpation of the lumbar and sacral ganglia on the abdominal and subcutaneous fat. Both the deposition and mobilization of fat are retarded, the latter more than the former. The same results were also found in the rat. The sympathetic nervous system thus has some regulating action on the fat depots. The same conclusion was reached by Hausberger (130). He cut the regional branches of the mixed nerves of

the interscapular fatty body; after starvation, this side contained more fat. No proof was given that these fibers were sympathetic. A further influence was shown by Kuré, Oi & Okinaka (131). They studied the subcutaneous fat after cutting the anterior or posterior roots. After cutting the anterior (sympathetic) roots, the fat deposition was increased. On the contrary, the cutting of the posterior roots, which they assume to contain parasympathetic fibers in accordance with Kuré's view (which, however, is not generally accepted), led to a decreased fat content. Cutting of a peripheral nerve in which both fibers are mixed influences both deposition and mobilization. Thus, the sympathetic would seem to mobilize and the parasympathetic to deposit fat. It should be noted, however, that Bezánák, whose paper was not known to the authors, had described an action of the sympathetic of both kinds.

The reviewer would like to express the opinion that, since choline has a fat-mobilizing action and acts generally like stimulation of the parasympathetics, one would expect that after section of the parasympathetics fat would be deposited. But the choline action on the liver might be different from that on the depots!

It is not yet clear whether calcium salts, given *per os* or subcutaneously or mobilized through the parathyroid hormone, act on fat metabolism *via* the sympathetic nervous system. But shortly after their increase, in the blood of the dog and man, the fat and cholesterol content of the blood also increase for about two to three hours (132).

Pathological inhibition of fat absorption.—Since it is now clear that fat absorption is regulated by the adrenal cortical hormone, an experimental basis can be found to explain the so-called "idiopathic steatorrheas." It was explained by Verzář (133) that the fat stools in Gee-Herter's disease and in the similar illness of the adult, the so-called "non-tropical sprue," might both be the result of an adrenal cortical insufficiency. There are many cases published which show, besides fat stools, pigmentation and adynamia. Such cases might be called "interrenal steatorrhea" or rather "interrenal absorption disturbances" since glucose absorption is also inhibited just as after experimental adrenalectomy. An experimental Gee-Herter's disease can be produced by the chronic poisoning of young rats with iodoacetic acid, which inhibits sugar and fat absorption, and also growth. Flavinphosphoric acid (from yeast and liver) cures the experimental illness just as effectively as in man.

Lately, it was emphasized by Pock-Steen (134), who did not know of these researches, that tropical sprue (Hill diarrhoea) also must be the result of adrenal insufficiency. There is a low blood sugar, low blood pressure, low muscle-tone—the general appearance being reminiscent of a hypopituitarism (Simmond's disease). That non-tropical sprue and Gee-Herter's disease are disturbances of fat absorption was again shown by Lawaetz & Vogt-Moeller (135); blood-lipid estimations with the "haemolipocrite" showed a diminished hyperlipaemia after fat ingestion by these patients.

The lipoidoses.—Much interest has been aroused lately by the study of the so-called "lipoidoses." These are illnesses in which enormous quantities of certain lipids are deposited in certain organs.

Epstein (136), who has worked for a long time in this field with much success, had shown that in Niemann-Pick's disease the total phosphatides of the spleen might increase about one hundred fold, and in the liver thirty-nine fold. He had also shown in 1932 that the phosphatide might be, besides lecithin, sphingomyelin. Furthermore, cholesterol, especially in the free form, was found in increased quantities in these organs.

Klenk (137) has lately published the following analyses which show a difference between the lipids of the brain in Niemann-Pick's disease and in Tay-Sachs idiocy, which was often thought to be related to the former:

	BRAIN LIPIDS			
	Glycerol Phosphatides	Total Protagons	Sphingo- myelin	Cerebro- sides
	Grams per 100 gm. of brain tissue			
Niemann-Pick's disease ...	17.6	27.6	8.9	0.34
Tay-Sachs idiocy	29.7	26.9	1.0	1.20

Thus, the quantity of sphingomyelin shows marked differences in the two diseases. Tropp & Eckhardt (138) found in a case of Niemann-Pick disease with Tay-Sachs idiocy much less sphingomyelin than in Klenk's earlier case. In a case observed by Teunissen (139) the spleen and liver showed a marked increase in lecithin, as also in sphingomyelin, which is in confirmation of Epstein. Also great quantities of free cholesterol were present.

In Gaucher disease, cerebroside, especially kerasin, are increased, as in Christian-Schüller disease, cholesterol. Epstein (140) now describes a new form of general cholesterol lipoidosis which he calls Bogaert-Scherer disease. Free cholesterol is deposited, especially

outside the cells. In the case in question there was 41.6 times more free than esterified cholesterol, while in Christian-Schüller disease the relation is opposite, there being four to ten times more of the esterified cholesterol, mostly intracellular.

One is obliged to believe that these illnesses are disturbances of intermediate lipid metabolism in which the transformation of phosphatides or of cholesterol esters is in some way inhibited. From the newer concepts of the influence of the adrenal cortex on the formation of phosphatides, Epstein believes that a change in cortical hormone production might explain these rare illnesses.

The enormous fat depositions sometimes observed under physiological and pathological conditions have been studied by Traina Rao (141).

Cholesterol metabolism.—The papers of Starkenstein and co-workers (142) on the physiology and pharmacology of the sterols give a good survey of the literature and problems in this field. Cholesterol seems to be only an end product of sterol metabolism. It is important as a factor in cell permeability, in absorption, and in narcosis. Saponins through precipitation disturb the equilibria in which cholesterol plays a part; cholesterol solvents have the same effect. Thus both increase the absorption of water-soluble substances from the stomach, skin, and bladder by influencing the lipid structure of the cell membranes of the epithelial cells. The same mechanism might also explain their pharmacological activity on muscle and the nervous system. If small amounts of cholesterol are injected, subcutaneously or intravenously, the narcotic dose of ether and other narcotics is lessened and also the narcosis so produced is deeper. The explanation offered is that the quantity of "transport-cholesterol of the blood" is increased. But also intravenously injected saponin increases the action of narcotics, a fact which is explained as being due to its mobilizing body cholesterol which then acts in the same way as does the injected cholesterol.

The production of cholesterol in the body was studied by Channon & Tristram (143) in continuation of researches on the effect of feeding rats with a diet containing 1 per cent of the hydrocarbon squalene. The cholesterol of the liver was increased about twofold, while carcass sterol was unaffected. The increase was in cholesterol esters. Hydrogenated squalene and *n*-hexadecane were not absorbed and no increase in liver cholesterol occurred. Feeding squalene to codfish produced no increase of liver cholesterol. It was suggested

that squalene is not converted into cholesterol and that the increase of the latter must be otherwise explained.

The very interesting method of studying sterol synthesis by injecting mice with heavy water led Schoenheimer & Rittenberg (73), as mentioned above, to the following conclusions: Cholesterol is synthesized through a process "which involves the coupling of a large number of small molecules." In contrast to what is found in the adult, experiments on chickens removed from the egg, where they had developed in a medium of heavy water, showed that cholesterol (just as fatty acids also) contained no deuterium. "This result," the authors say, "excludes the occurrence of cholesterol synthesis during development."

There is some discussion on the influence of the gonads on cholesterol metabolism. It is believed (144) that castration influences cholesterol metabolism but the experimental proof is rather weak. Blood cholesterol increases in cows during pregnancy (145). In the blood of the placental vein and artery, no difference in cholesterol content could be shown, but there was certainly less than in the maternal blood.

Bile or bile salts inhibit the esterification of free cholesterol in human and monkey blood serum and, in low concentrations, also in dog serum. High concentrations give uneven results (146). From the work of Riegel and coworkers (147), one might explain this in a somewhat different way. Riegel *et al.* incubated blood plasma and the cholesterol was then esterified. If bile was added, the cholesterol esters were hydrolyzed. Some bile contains cholesterol esters, and such bile does not hydrolyze. It might, therefore, not contain the necessary esterase. In this connection we might refer again to Boyd's work (18) showing a breakdown of cholesterol esters in blood plasma during prolonged preservation.

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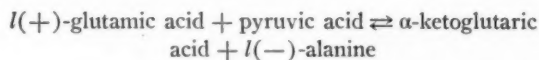
METABOLISM OF AMINO ACIDS AND PROTEINS*

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GENERAL ASPECTS OF AMINO ACID METABOLISM

Intermolecular transfer of amino groups.—One of the most important contributions to the subject is the discovery of the intermolecular transfer of amino groups (1). The authors show that the following reversible reaction takes place in animal tissues:



The reaction was found to occur in muscle, heart, brain, liver, and kidney. α -Ketonic acids other than pyruvic acid, for example, α -ketobutyric, α -ketocaproic, and oxaloacetic acid, also may serve as acceptors for the amino group of glutamic acid; on the other hand α -amino acids readily give up their amino group to α -ketoglutaric acid in the presence of muscle tissue. The formation of glutamic acid has been demonstrated with sixteen different natural and racemic amino acids including glycine and histidine. The rate of the reaction is astonishingly high; thus no less than 6.5 mg. glutamic acid are formed in two minutes from glycine and α -ketoglutaric acid by 1 gm. of muscle tissue. Aspartic acid may replace glutamic acid and oxaloacetic may replace ketoglutaric acid, as amino nitrogen donor and acceptor respectively, but no transfer of the amino group occurs unless either the amino acid or the α -ketonic acid is a dicarboxylic acid.

The great velocity of these reactions suggests that they play an important role in cellular metabolism but it is as yet too early to define this role in detail. These findings explain the results of Needham (2) who observed that muscle can remove glutamic acid without liberation of ammonia.

Behaviour of stereoisomerides.—The view that the amino acids of the *d*- and *l*-series are broken down in the animal body under the influence of two different specific enzymic systems (3) has been

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confirmed by the work of Felix *et al.* (4), quoted in the sections on tyrosine and phenylalanine. Several papers demonstrate again the utilization of the amino acids of the *d*-series and their conversion into the corresponding enantiomorphs of the *l*-series in the animal body. Conrad & Berg (5) fed rats on a histidine-deficient diet supplemented with *d*(+)-histidine and found the histidine in the tissue of these animals to be pure *l*(-)-histidine. Kotake & Goto (6) report similar experiments with tryptophane using for the determination of *l*(-)-tryptophane a bacterial enzyme which converts *l*(-)-tryptophane, but not *d*(+)-tryptophane, into indole (7, 8). They showed the conversion of *d*(+)-tryptophane into *l*(-)-tryptophane to occur *in vitro* in slices of liver or kidney. Whipple & Robscheit-Robbins (9) showed that *d*(+)-histidine and *d*(+)-tyrosine are utilized by the anaemic dog for the regeneration of haemoglobin to the same extent as are the antipodes of the *l*-series.

Occurrence of the amino acids of the d-series.—Bruckner & Ivánovics (10, 11) discovered a polypeptide containing *d*(-)-glutamic acid (as the only amino acid) in bacteria-free culture mediums of *Bacillus anthrax* and other spore-forming bacteria belonging to the *mesentericus* group. The copper salt of the polypeptide is sparingly soluble in water and it can be used as a starting material for preparing *d*(-)-glutamic acid. The polypeptide proved to be identical with the specific haptene of *Bacillus anthrax* and the authors suggest that it is the function of this specific protein in the capsule of the bacilli to protect the organism against the proteolytic enzymes of the host. Proteolytic enzymes, as is well known, only attack polypeptides built up from amino acids of the *l*-series.

Role of d-amino acid deaminase.—Weil-Malherbe (12) found that purified preparations of "succinic dehydrogenase" attack *d*(-)-glutamic acid only, whilst the starting material, muscle tissue, oxidizes *l*(+)-glutamic acid only. The same change of optical specificity was found for the "glutamic acid dehydrogenase" of brain. These findings support the view (3) that *d*-amino acid deaminase is a fragment of *l*-amino acid deaminase.

Metabolism of methylated amino acids.—Whilst tryptophane (13) and histidine (14) may be replaced in the diet by their α -N-monomethyl derivatives, it is not possible, according to Gordon (15), to replace lysine by α -N-monomethyl lysine or α -N-dimethyl lysine in the diet of growing rats. These differences may be due to the peculiar specificity of the deaminating enzymes. Keilin & Hartree (16)

showed that some α -N-monomethyl amino acids, e.g., methyl alanine, are oxidized to the ketonic acid, whereas others, e.g., N-monomethyl tyrosine, are not attacked.

Acetyl amino acids.—Du Vigneaud *et al.* (17) found that acetylation of the laevorotatory homocystine neither affects appreciably its availability for growth purposes nor to any great degree its oxidation *in vitro* whereas acetylation of dextrorotatory homocystine prevents its utilization for growth purposes and hinders greatly its oxidation. This result is to be expected if the laevorotatory form is the isomer belonging to the *l*-series, since animal tissues hydrolyze the acetyl compounds of the *l*-series only (18, 19). This "biological" determination of the spatial configuration of the optical isomers confirms the conclusions drawn previously from chemical evidence.

Deamination of amino acids with branched chains.—Corley & Snyder (20) administered to a dog, parenterally, amino acids with branched chains and analyzed the urine for total nitrogen, urea nitrogen, and amino acid nitrogen. They came to the following conclusions: (a) A methyl group attached to the carbon atom carrying the amino group interferes with deamination (see also 20a); (b) a methyl group attached to the carbon atom, the second from that carrying the amino group, does not interfere with deamination; (c) a methyl group attached to the carbon atom next to that carrying the amino group does not interfere with deamination if the amino acid has the natural *l*-form, but does interfere with deamination of the amino acid with the antipodal form.

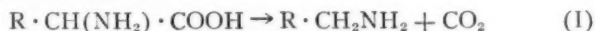
The first two points are in complete accordance with the work on isolated tissues and enzyme systems (see 3, 16); the third point has not yet been studied *in vitro*.

Site of deamination.—That kidney, in addition to liver, plays a role in the deamination of amino acids (3) has been confirmed by Oberdisse & Eckhardt (21) who worked with the isolated heart-lung-kidney preparation of Starling & Verney. Basu & Basak (22) examined the deamination of thirteen amino acids in isolated rat heart and rat lung. Heart attacked *l*(—)-cystine and *l*(—)-proline only, and the rate of deamination was very slow. Lung tissue deaminated *l*(—)-cystine only (see also 22a). Burns and Cruickshank (23) claim to have shown that the excised mammalian heart, under "aglycaemic" conditions (i.e., if deprived of carbohydrate) can produce urea and utilize glycine. Their figures are, however, not very convincing. More convincing are experiments by Clark, Gaddie & Stewart (24) on the frog

heart, which seems to be capable of utilizing glutamic acid and alanine.

Glycogenesis from amino acids.—Quantitative data about the glycolytic and ketolytic properties of *l*(+)- and *dl*-glutamic acids, *l*(-)- and *dl*-aspartic acids, and *dl*-pyroglutamic acid are reported by Butts, Blunden & Dunn (25). The most effective amino acid was *l*(-)-aspartic acid, followed in turn by *dl*-aspartic acid, *dl*-pyroglutamic acid, *l*(+)-glutamic acid, and *dl*-glutamic acid. Bach & Holmes (26) found *in vitro* a gluconeogenesis from alanine, aspartic acid, glutamic acid, and arginine in slices of liver of starved rats. Less glycogen was formed if insulin was added to the slices *in vitro* and the authors conclude that insulin acts in these experiments by inhibiting synthesis of carbohydrates from amino acids.

Formation of amines from amino acids.—Whilst it has long been known that bacteria can decarboxylate certain amino acids to form the corresponding amine:



it was doubtful whether, or to what extent, animal tissues bring about reaction I. Last year Bloch & Pinösch (27) and Werle (28) presented evidence suggesting the conversion of histidine into histamine. Holtz (29, 30) now claims to have found a formation of histamine from histidine in the kidney and liver of rabbits and guinea pigs, but not regularly in those of cats. Positive results were obtained by Holtz only in anaërobic experiments, probably because histaminase destroys histamine when oxygen is available.¹ Holtz (32) also obtained tyramine from tyrosine (2 mg. in twelve hours from 2 gm. tissue in the presence of rabbit and guinea-pig liver and kidney) whilst experiments with cat and dog kidney were negative. All conclusions of Holtz are based on the pharmacological tests. The formation of tyramine has also been studied by Heinsen (33) who found in ox pancreas a "tyrosine decarboxylase" which catalyzes the conversion of tyrosine into tyramine. The enzyme was present in minced pancreas and to a lesser extent in dried pancreas, but not in extracts. The tyramine formed was isolated as benzoate; for instance, 3.4 gm. benzoate were found in an experiment in which 200 gm. pancreas were incubated with 5 gm. *l*(-)-tyrosine for eight days. The rate of the decarboxylation is thus very slow as com-

¹ This may explain the controversial results of Zipf & Gebauer (31).

pared with the rate of the decomposition of tyrosine in liver or kidney. The tyrosine decarboxylase was not detectable in muscle, kidney, spleen, and intestine, and contrary results published by Schuler *et al.* (34, 35) are considered by Heinsen (33) as based on unsatisfactory methods. It becomes evident from the work reported that reaction I is a side reaction in animal tissues. Only a few special amino acids may react in this way, but even the bulk of histidine and tyrosine in the animal body follows other pathways of catabolism.

Okunuki (36) described in plants a "glutamic decarboxylase" which brings about the conversion of *l*(+)-glutamic acid into δ -aminobutyric acid. The enzyme was found in the dried powder of beets and carrots and of the bulbs and pollen of *Lilium auratum*. It was absent from potatoes, onions, beans, barley, apples, pears, and brewer's yeast. Glutamic and pyrrolidone dicarboxylic acids were the only compounds yielding carbon dioxide. Many amino acids tested did not react.

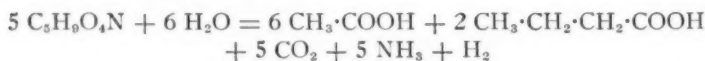
Virtanen & Laine (37), working with *B. coli* and nodule bacteria, studied in detail the decarboxylation of *l*(+)-lysine and *l*(-)-aspartic acid according to I. Some strains of the organisms decomposed the amino acid almost quantitatively, yielding cadaverine and β -alanine respectively. The pH optimum was about 7.0. The reaction is accomplished only by living bacteria.

Intermediary links between carbohydrates and amino acids.—Until recently pyruvic acid was considered to be the chief link between carbohydrates and amino acids; it alone was known to arise from both groups of foodstuffs and to be convertible into either carbohydrate or amino acid. The recent work on the intermediary breakdown of carbohydrate has revealed similar key positions for oxaloacetic and α -ketoglutaric acids. These two ketonic acids arise in the course of the breakdown of sugar in animal cells and probably also in plants (38, 39, 40, 41). Their relations to aspartic acid and glutamic acid have long been known. Since living cells can synthesize amino acids by reductive amination of the corresponding ketonic acid, it becomes clear whence the cells may obtain the carbon skeleton for the synthesis of glutamic and aspartic acids. In view of the fact that glutamic acid is related in intermediary metabolism to proline (42, 43) and to histidine (44), α -ketoglutaric acid is of special importance as a bridge between carbohydrates and proteins.

Several workers have shown in recent years the special role played by glutamine and asparagine in the metabolism of certain plants (41,

45, 46, 47). These amides are formed in large amounts if the plants are grown in the presence of much ammonium salt, and the formation of the amide has been interpreted as a detoxication (48). Since the carbon skeletons of these amides, being normal intermediates in carbohydrate breakdown, are more abundantly available than any other analogous substances, it is easy to understand why the cells choose to synthesize glutamine and asparagine for the binding of ammonia.

Bacterial deamination.—Woods & Clifton (49) investigated the breakdown of amino acids by a strictly anaërobic bacterium, *Clostridium tetanomorphum*, and discovered a mode of breakdown of amino acids not hitherto described for any type of cell: Amino acids, in the presence of *Clostridium tetanomorphum*, yielded carbon dioxide, ammonia, and hydrogen. The amino acids decomposed in this way are *l*(+)-glutamic acid, *dl*-serine, *l*(-)-aspartic acid, *l*(-)-histidine, *l*(-)-cysteine, *l*(-)-cystine, *l*(-)-tyrosine, *l*(-)-methionine, whilst glycine, alanine, leucine, arginine, lysine, and others are not attacked. The organism does not form hydrogen from formate, and formate cannot therefore be an intermediate in the breakdown of amino acids. In the case of glutamic acid, the end-products, in addition to carbon dioxide, ammonia, and hydrogen, are acetic and butyric acids and their quantities suggest the following balance sheet for the decomposition of *l*(+)-glutamic acid:



In the case of the other amino acids the yields of carbon dioxide, ammonia, and hydrogen were measured but the complete balance sheets were not determined. *B. coli*, in contrast to *Clostridium*, does not deaminate amino acids anaërobically (50) but does so in the presence of oxygen. The aerobic deamination of glycine, *dl*-alanine, and *l*(-)-aspartic acid in *B. coli* has been studied in detail by Stephenson & Gale, that of glycine by Janke & Tayenthal (51).

The work on coupled reactions between pairs of amino acids in *Clostridium sporogenes*, initiated by Stickland in 1935 (51a), has been extended by Woods (52) who found that *l*(+)-arginine and *l*(+)-ornithine are activated as hydrogen acceptors and *l*(-)-cysteine as hydrogen donor in these reactions. When ornithine reacts with the donor, it undergoes reductive deamination to δ -aminovaleric acid.

Metabolism of amino acids in moulds.—Van Waesberghe (53) studied the breakdown of asparagine in *Aspergillus niger*, *Ascochyta pisi*, *Mucor racemosus*, *Fusarium conglutinans*, and *Cylindrocarpum radiciola*. "Impoverished" mycelium was used for the experiments. Ammonia was liberated in acid (pH 4.0) and alkaline media (pH 8.81). The hydrolysis of the amide group ("asparaginase"), as in animal tissues and in yeast, was independent of the presence of air, whilst the deamination of the α -amino group occurred in the presence of oxygen only. Sugar is preferentially oxidized by *Aspergillus* and prevents the breakdown of asparagine. Only when the nutritive reserves are exhausted, therefore, does the deamination and deamidation of asparagine set in. The oxidation of asparagine by *Aspergillus* is incomplete. In acid medium three atoms of oxygen are absorbed for each molecule of aspartic acid added; in alkaline medium, 0.8 to 2.0 atoms are absorbed.

Jacquot (54) finds that *Aspergillus niger*, if grown on a medium containing potassium nitrate as the only nitrogenous nutrient, forms considerable quantities of urea and of ammonia. The mechanism of this urea synthesis has not yet been studied.

METABOLISM OF INDIVIDUAL AMINO ACIDS

Glycine.—Adams *et al.* (55) studied the effect of administration of glycine on the urinary excretion of nitrogenous substances in man. Confirming previous work, they find an increased excretion of uric acid and of creatine.

Glutamic acid.—Euler *et al.* (56) prepared a specific "glutamic dehydrogenase" from bottom yeast. The enzyme requires "codehydrogenase II" (but not cozymase) as coenzyme. Warburg's flavoprotein acts as a carrier in the system between the acceptor (oxygen or methylene blue) and dihydrocohydrogenase II.

Canavanine and canaline.—An excellent review of the chemistry and biochemistry of canavanine and canaline has been published by the discoverer of these amino acids (57).

Tyrosine.—Felix *et al.* (4) (see also 58) in a study of the breakdown of tyrosine by minced liver tissue find that the additional oxygen absorption induced by *l*(-)-tyrosine depends on the pH. The extra oxygen uptake per molecule of *l*(-)-tyrosine was: one atom at pH 6.8, two atoms at pH 7.2, and three atoms at pH 7.6 to 7.8. These findings may explain some controversial results of Bernheim (59) and of Edson (60) (see also 3) published previously. One

molecule of acetoacetic acid and one molecule of carbon dioxide appear as end-products of the oxidation whilst no ammonia or urea is formed. *p*-Hydroxyphenylpyruvic acid yields the same end-products and absorbs three atoms of oxygen, but since no ammonia is formed during the oxidation of tyrosine and since the authors failed to detect *p*-hydroxyphenylpyruvic acid among the products of the oxidation of *l*(-)-tyrosine the authors suggest that *p*-hydroxyphenylpyruvic acid is not an intermediate in the breakdown of tyrosine in liver. This argument is, however, not conclusive, especially in view of the work of Braunstein & Kritsman (1) on the transfer of amino groups.

Felix *et al.* (4) state, furthermore, that *l*(-)-tyrosine absorbs one atom of oxygen in kidney tissue without liberation of ammonia; *d*(+)-tyrosine, in contrast to *l*(-)-tyrosine, yields ammonia and *p*-hydroxyphenylpyruvic acid in liver and kidney.

Phenylalanine.—According to Felix *et al.*, *l*(-)-phenylalanine absorbs one atom of oxygen in liver or kidney without liberating ammonia or a ketonic acid, whereas *d*(+)-phenylalanine yields the theoretical quantity of ammonia and a certain amount of phenylpyruvic acid. Part of the phenylpyruvic acid formed appears to be oxidized to phenylacetic acid and carbon dioxide.

Histidine.—Abderhalden & Hanson (61) confirm Edlbacher's (44) discovery of the conversion of histidine into *l*(+)-glutamic acid under the influence of histidase. A number of papers deal again with the occurrence of histidine or a related compound in the urine ["imidazoluria" (62)]. Schimmelpfeng (63) found histidinuria in cases of organic nervous diseases and also of functional disorders, especially in melancholia. Norpoth (64) observed histidinuria in cases of acromegaly, Cushing's syndrome, and adiposogenital dystrophy, and points out that the anterior pituitary appears to play a special role in histidine metabolism. The histidinuria of pregnancy may also be due to dysfunction of the hypophysis.

Breakdown of histidine in model systems.—Edlbacher & Segesser (65) and Holtz *et al.* (29, 66, 67, 68) discovered independently that histidine undergoes decomposition *in vitro* if treated with ascorbic acid, or sulphhydryl compounds (glutathione, cysteine, thioglycollic acid) in the presence of oxygen and of traces of iron (neutral medium; 38° C.) (see also 69). Up to 80 per cent of the histidine nitrogen appears as ammonia after alkalisation. Thus there must be a decomposition of the imidazole ring, but the mechanism of this

reaction is different from the action of histidase. The latter is a hydrolytic reaction whereas the decomposition in the presence of sulphydryl compounds or ascorbic acid requires oxygen.

Imidazolelactic acid, methylhistidine, histamine, imidazole, hypoxanthine, adenine, and carnosine react similarly to histidine in the model. Holtz & Heise (68) find that histamine is formed from histidine in the model under suitable conditions. Histamine was also formed when an aqueous solution of histidine was treated alternately with hydrogen and oxygen in the presence of palladium (70).

Tryptophane.—Böhm (71) administered a great number of indole derivatives to men and animals and examined the excretion of indican in the urine. Confirming older work, he found that only those indole derivatives which have no substituents in positions 2 and 3 would yield indican. Indole derivatives with substituents in position 2 yield colourless substances which form red pigments when tested with Obermeyer's or Rose-Exton's reagent. Indole derivatives with substituents in position 3 probably form indolacetic acid.

Both optical isomerides of tryptophane are broken down in the rabbit in the same manner, *l*(—)-tryptophane yielding *l*(—)-kynurenine, and *d*(+)-tryptophane yielding *d*(+)-kynurenine (72).

PEPTIDES AND POLYPEPTIDES

Larizza (73) published a series of papers on the "metabolism of polypeptides" in blood. He studied the difference between the non-protein nitrogen content of the trichloroacetic acid and the tungstic acid filtrates of blood and assumes that this difference is due to polypeptides which are precipitated by tungstic but not by trichloroacetic acid. The difference was, on the average, 2.21 mg. per cent in human serum and 4.2 mg. per cent in human blood; it was normal in disorders of the liver, but increased in leucaemia, carcinoma, pneumonia, severe tuberculosis, and renal diseases.

The mode of breakdown of glutathione has been investigated by Rosenbohm (74) who concludes from experiments based on the Sullivan test that kidney extracts ("antiglyoxalase") form glutaminylcysteine from glutathione whilst cysteinylglycine is formed in other tissues. The author claims that it is possible to distinguish the various sulphydryl compounds by the intensity of colour in the Sullivan test.

Carnosine was found to support the growth of rats on a histidine-

deficient diet (75) and this result has been taken to indicate that the peptide can be hydrolyzed in the tissues.

PROTEINS

Blood-plasma proteins.—Whipple and his collaborators (76, 77, 78) continue their important studies on blood-plasma-protein regeneration. The blood-plasma proteins in these investigations are depleted by bleeding and return of the washed red cells. With this procedure it is possible to bring the dog to a steady state of low plasma protein in the circulation and a uniform plasma-protein production on a basal diet. These dogs become test subjects by which the effect of various factors on plasma-protein regeneration can be measured.

A reduction in plasma-protein regeneration is induced by a sterile abscess (turpentine) (78) or by digestive disturbances (76). Iron, liver extract, or thyroid powder has no effect. The proteins of red blood cells when added to the diet are poorly utilized. Kidney proteins show a higher potency than blood cells and fresh serum is the most efficient source of protein. An Eck-fistula dog, shown by biopsy to have an abnormal liver, had less than one-tenth of the capacity of the normal dog to form new plasma protein when various food proteins were added to the basal diet (77). This observation gives strong support to the view that the liver is actively concerned with the production of plasma protein.

Haemoglobin.—Pearson, Elvehjem & Hart (79) investigated, in rats, whether the quantity and level of proteins are factors in the rate of haemoglobin regeneration in nutritional anaemia, when the intake of iron and copper is adequate to permit optimal response. Rats were made anaemic by feeding on a diet consisting exclusively of milk and the effect of nine different proteins on haemoglobin regeneration was studied. The proteins of liver, casein, egg albumin, and soybean meal were consistently effective in building up haemoglobin. No specific haematopoietic property could be ascribed to any of the materials. Corn gluten meal, wheat gluten, gliadin, and gelatin were poorly utilized for the formation of haemoglobin.

Bence-Jones protein.—Vignati & Rauchenberg (80) describe a case of myeloma in which they found enormous amounts of the Bence-Jones protein in the serum but only traces in the urine. The serum coagulated at 49° and contained 167.8 gm. total protein and 140 gm. globulin per liter.

A number of other papers can here be mentioned only under

general headings: Several (81, 82, 83) deal with blood serum and plasma proteins. Terroine and his school published a series of papers on the "regulation" and the pharmacology of nitrogen metabolism (84, 85, 86). The non-protein nitrogen of the blood under various conditions is also the subject of report (87, 88, 89).

AMMONIA FORMATION IN TISSUES

Experiments of Conway & Cooke (90) show a peculiar distribution of the purine deaminases in different tissues. Adenosine is rapidly deaminated in shed and lysed blood of man, fowl, frog, and the lug worm (*Arenicola*). The deaminating enzyme is present in the plasma and in the cells. Muscle adenylic acid is practically untouched by this adenosine deaminase, whilst muscle, as is well known, contains a very active adenylic acid deaminase. The activity of the enzyme in rabbit blood is so high that one is led to suppose that all the adenosine deaminase previously found in muscle and liver may really have been contained in the residual blood in the tissues.

The nucleated corpuscles of the fowl were found to deaminate also adenine, guanine, and cytosine. Engelhardt & Baev (91) report that washed intact avian blood cells form no ammonia from endogenous sources as long as their respiration is intact; a rapid formation of ammonia sets in if respiration is prevented.

UREA

Urea synthesis from ammonia.—Gorter (92) confirmed the stimulating effect of ornithine on the rate of urea synthesis in liver slices. London & Alexandry (93), on the other hand, state that they were unable to find an effect of ornithine on the rate of urea synthesis in the angiotomised dog after intraportal injection. The reviewer, however, is unable for the following reasons to accept London's experiments as evidence against the "ornithine cycle" *in vivo*:

(a) London determined the concentration of urea in the blood of the hepatic vein, but not the rate of blood flow and therefore not the rate of urea formation. Conclusions about the intermediate mechanism of metabolic processes can only be drawn from experiments in which the rates of reaction have been measured.

(b) London *et al.* state that they used ornithine dihydrochloride. This substance is strongly acidic and it is therefore not surprising that it had not the expected effect, and that it even inhibited urea formation in some of London's experiments.

(c) The unsuitability of London's experimental conditions is also shown by the fact that in his experiments arginine yielded even less urea than ammonium chloride although it is well known that arginine is very rapidly decomposed into ornithine and urea (94). A possible explanation for London's results is the use of the alkaline arginine base, instead of the neutral arginine monohydrochloride.

(d) It is certain that ornithine promotes the urea synthesis in isolated tissue *in vitro*, and it is therefore very difficult to assume that this highly specific mechanism is not present in the intact tissue *in vivo*, but is only produced by the special "abnormal" conditions of the *in vitro* experiment.²

Urea formation from purines.—Brunel (95), confirming the work of Przylecki (96), Stransky (97), and Krebs & Weil (98), studied the metabolism of purines in fish. He finds that in the liver of *Raja*, a selachian, the pathway of the breakdown is the same as previously

shown for the frog (99): Uric acid $\xrightarrow{\text{(uricase)}}$ allantoin $\xrightarrow{\text{(allantoinase)}}$ allantoic acid $\xrightarrow{\text{(allantoicase)}}$ urea + glyoxylic acid (II). He studied the specificity of allantoicase and also found its pH optimum, in accordance with Krebs & Weil (98), to be near the neutral point. Some teleosts (*Esox*, *Cyprinus*, *Leuciscus*, *Scomber*) possess the same uricolytic enzymes as *Raja*; others, however (*Salmo*, *Pleurohacter*, *Rhombus*, *Mullus*, *Zeus*, *Gadus*, *Merlangus*, *Conger*, *Anguilla*), do not possess allantoicase and therefore yield allantoic acid as end-products of purine metabolism.

Fosse and his collaborators continued their work on the metabolism of purines and ureides in plants and found a number of other species in which the series of reactions (II) occur. Leroux (100) found allantoic acid, previously shown to occur in the leaves of *Acer pseudoplatanus*, *Phaseolus vulgaris*, and other Leguminosae (cf. also 101), in *Coryllus avellanus* (0.43 gm. per 100 gm. dry leaves). Echevin & Brunel (101) quantitatively investigated the occurrence of purines and ureides in the beans and the germinating plants of *Soja hispida* and also the relative enzymic activity of uricase, allantoinase, allantoicase, and urease in this plant. Brunel demonstrated the presence of uricase and allantoinase in fungi (103) and higher plants (*Agrostemma githago*) (104).

² The suggestion that experiments *in vitro* do not allow us to draw any conclusions about metabolic processes in the intact body has also been considered and refuted by Meyerhof (134) and by Parnas (135).

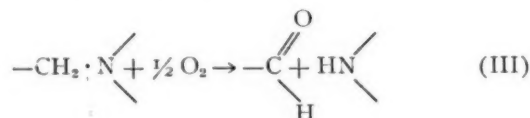
URIC ACID SYNTHESIS IN BIRDS

Reindel & Schuler (105) claim to have identified xanthine as an intermediate in the synthesis of uric acid from ammonia in the pigeon. This is contrary to the previous results of Edson, Krebs & Model (106) who had isolated hypoxanthine. Experiments carried out more recently by the reviewer (19) confirm the previous results and make it certain that the primary product in the uric acid synthesis from ammonia is hypoxanthine. The new results were obtained with a new method of determining and distinguishing small quantities of the purine bases: The purines are oxidized under the influence of xanthine oxidase and the amounts of oxygen required and uric acid formed are measured; the ratio " O_2 used" / "uric acid formed" is 1.0 in the case of hypoxanthine and 0.5 in the case of xanthine, and a given ratio allows us to calculate the concentrations of hypoxanthine and xanthine in the solution. Pigeon liver formed small quantities of xanthine on autolysis, probably from guanine contained in the nucleic acids, but the purine base formed synthetically in liver was found to be hypoxanthine.

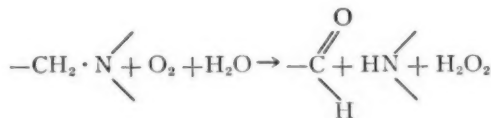
OXIDATION OF AMINES INCLUDING ADRENALINE AND RELATED SUBSTANCES

The pioneer work of Guggenheim & Löffler (107) on the fate of amines in the animal body has been extended by a series of papers dealing with the enzymic oxidation of amines *in vitro*. Up to 1937, histamine, adrenaline, and tyramine were the only amines (apart from amino acids) the biological oxidation of which had been studied in detail by modern methods. The new work shows that a number of animal tissues, in the form of slices or of extracts, are capable of oxidizing amines. Pugh & Quastel (108) found an oxidation of butylamine, amylamine, isoamylamine, and heptylamine in brain slices and extracts. The oxidation of lower amines, propylamine, ethylamine, and methylamine was less marked or negligible. Similar results were obtained with rat liver and guinea-pig liver or kidney. A detailed study of the amine oxidase was made by Philpot (109) and by Kohn (110) working chiefly with tyramine, and by Richter (111) and Blaschko, Richter & Schlossmann (112) working chiefly with adrenaline and related compounds. It appears from this work that the oxidation of the aliphatic amines, tyramine, tryptamine, adrenaline, phenylethylamine, dimethylamine, benzylamine, *dl*-arterenol, epinine, *l-p*-sympatol, and hordenine (111, 112) is due to one and

the same enzyme, which is, however, different from amino acid oxidase and different from histaminase. Richter (111) isolated the products of oxidation and found that they were, in each case, an aldehyde and ammonia or a lower amine. The reaction may be represented by the general equation (111, 112):



With regard to the mechanism of this reaction, the important points are: (a) hydrogen peroxide is formed, as shown by the coupled oxidation of alcohol in the presence of peroxidase (108, 109); (b) molecular oxygen can be replaced by certain dyes (*o*-bromophenolindophenol, *o*-cresolindophenol) (109); (c) the list of substrates comprises primary, secondary (adrenaline), and tertiary (hordenine) amines. These facts suggest the following mechanism (111, 112):



In the presence of catalase the reaction $2 \text{H}_2\text{O}_2 = 2 \text{H}_2\text{O} + \text{O}_2$ follows and the balance sheet is that of reaction III.

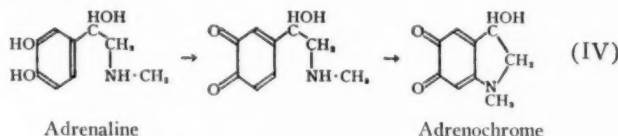
The enzyme is cyanide-insensitive, but is inhibited by octyl alcohol and by ephedrine. The enzyme also occurs (112) in echinoderms (*Asterias rubens*, *Echinus esculentus*) and molluscs (*Patella vulgata*). Histaminase, on the other hand, is cyanide-sensitive; furthermore, its occurrence does not run parallel with the occurrence of amine oxidase and it must therefore be different from amine oxidase. The identity of the enzymes responsible for the oxidation of adrenaline, tyramine, and other amines is suggested (a) by their parallel distribution in the animal kingdom, (b) by their identical behaviour towards inhibitors, (c) by the fact that the different substrates compete with each other if present together (108, 112).

The older observations of Guggenheim & Löffler (107), who found *p*-hydroxyphenylacetic acid formed from tyramine in the

perfused liver, are now to be explained by a secondary dismutation of the aldehyde formed primarily.

Whether amine oxidase is connected with the formation of trimethylamine oxide found in the excretions of many organisms remains to be studied.

Adrenaline and related "catechol compounds," e.g., epinine, arterenol, corbasil, colephrine, can be broken down by animal tissues in two different ways. In addition to III, which oxidizes the adrenaline molecule in the side chain, there is a second reaction, IV, which, according to Green & Richter (113), oxidizes adrenaline in the following manner:



Catalysts bringing about IV are (a) the cytochrome-indophenol oxidizing system present in all tissues; (b) catechol oxidase from *Agaricus*; (c) a cyanide-insensitive system in heart and skeletal muscle; (d) haematin compounds such as haematin or methaemoglobin. The end-product of the oxidation was isolated (113) and found to be $C_9H_9O_3N$. It crystallizes in red needles and has the properties of N-methyl-2, 3-dihydro-3-hydroxyindole-5, 6-quinone (see formula in IV). The authors propose the name adrenochrome for this substance. Adrenochrome can be reversibly reduced and oxidized and can act as a respiratory carrier.

INBORN ERRORS OF METABOLISM

Tyrosinosis.—Blatherwick (114) examined more than 26,000 specimens of urine for tyrosinosis without finding a case. This reveals the extreme rarity of this error of metabolism.

Phenylketonuria.—Penrose & Quastel (115) continued their metabolic studies in phenylketonuria. Using a new rapid and reasonably accurate method for the determination of phenylpyruvic acid they found that *dl*-phenyltyrosine, *l*(-)-phenyltyrosine, and *d*(+)-phenyltyrosine lead to an increased excretion of phenylpyruvic acid in phenylketonuria. Tyrosine appeared to cause a slight increase in the concentration of the ketonic acid in the urine of phenylketonurics. The extra phenylalanine ingested by phenylketonurics was not all

converted into phenylpyruvic acid. In normal controls *d*(+)- or *dl*-phenylalanine produced a slight ketonuria whereas *l*(-)-phenylalanine did not lead to phenylketonuria. The results are in agreement with the view discussed previously (3) that phenylalanine undergoes two modes of breakdown in the normal body, (a) through phenylpyruvic acid, (b) through tyrosine, and that the fault in phenylketonuria does not lie in excessive conversion of phenylalanine into phenylpyruvic acid but in the failure to break down phenylpyruvic acid further.

Jervis (115a) describes the results of a clinical study of 50 cases of phenylketonuria ("phenylpyruvic oligophrenia"); 42 cases were found among 8043 inmates of an institution for mental defectives. The genetic data seemed to indicate that the condition is determined by a single recessive gene substitution. Of the cases 71 per cent were idiots and 29 per cent imbeciles. Phenylpyruvic acid was absent from the cerebrospinal fluid.

Cystinuria.—Beumer & Wepler (116) describe another of the rare cases of severe cystinuria in children which end fatally owing to deposits of cystine in many tissues and subsequent degenerative changes in the tissues, especially the kidneys. By feeding rats with large amounts of cystine or cysteine they succeeded in producing degeneration of the kidney tissue similar to the alterations found in human cases.

Brand, Block, Kassell & Cahill, continuing their metabolic studies on cystinurics (117, 118, 119, 120, 121), find that methionine and cystine, fed as constituents of casein and lactalbumin, are catabolized both qualitatively and quantitatively in the same way as when they are administered in the form of the free amino acids. The catabolism of carboxymethyl-S-cysteine, $[\text{HOOC} \cdot \text{CH}_2 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}]$ (I), in both normals and cystinurics leads to the excretion of a mixed disulphide $[\text{HOOC} \cdot \text{CH}_2 \cdot \text{S} \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}]$ (II). Substance I is apparently the source of the thioglycollic acid moiety of II, but the cysteine moiety of II may be furnished either by I or by cysteine available in the body. These results support the hypothesis suggested for the conversion of methionine into cysteine (see 3). Brand *et al.* (121) furthermore studied the behaviour of the hydroxy analogue of methionine (*dl*- α -hydroxy- γ -methiobutyric acid) in a cystinuric. The compound, like methionine, was largely excreted as extra cystine; it supports the growth of rats on a sulphur-deficient diet. This represents another example of the

general rule that essential amino acids can be replaced by the hydroxy- (or ketonic) derivatives. S-methyl cysteine, γ -thiobutyric acid, and γ,γ' -dithiodibutyric acid did not yield extra cystine in cystinuria (121).

Some further papers (122, 123, 124, 125) on the metabolism of cystine cannot be discussed in detail in this article.

HIPPURIC ACID

Before 1937 the synthesis of hippuric acid by animal tissues could be studied only in the intact body or in the perfused organ (126). Griffith (127) has now shown that surviving liver slices form hippuric acid *in vitro*, if benzoic acid and glycine are added, and Waelsch & Busztin (128) found that the synthesis of hippuric acid occurred when minced kidney or liver (horse) were incubated *in vitro* with benzoic acid and glycine. Benzoic acid and ammonia, under the same conditions, gave rise to the formation of benzamide. The authors studied the synthesis of benzamide under various conditions and found an inhibition by cyanide and an acceleration by reduced glutathione and cysteine. The enzymic system was active in glycerol extracts, but not in dried tissue or in aqueous extracts.

CLINICAL WORK

Kirk (130) has published an excellent monograph on amino acid and ammonia metabolism in liver disease (146 pages). It contains a critical review of previous work together with much original experimental clinical work. The author has improved previous methods and worked out new methods for the functional test of amino acid metabolism (blood amino nitrogen following ingestion of glycine) and urea synthesis (determination of the amount of urea formed after ingestion of ammonium citrate, "ammonia-tolerance test").

Blood ammonia was increased in liver cirrhosis (this confirms results of previous workers). Whilst normal persons showed no significant increase of the blood ammonia after ingestion of 10 gm. of ammonium citrate, there were increased blood ammonia values in most cases of liver cirrhosis after the ingestion of ammonium citrate. At the same time the urea synthesis appeared to be normal in liver cirrhosis and the author suggests, therefore, that the increased blood ammonia may be due to the existence in liver cirrhosis of a collateral portal circulation (129, 130).

Gjessing continued his metabolic studies in cases of mental dis-

orders. His new paper (132) is a careful investigation of a case of periodically recurrent catatonic mania. He finds, as in the cases of periodically recurrent catatonic stupor (131), periodic variations in the nitrogen balance. Shortly before the beginning of the period of mania there is an increased nitrogen excretion (negative nitrogen balance) which continues during the first half of the period of excitement. It is followed by a nitrogen retention (positive nitrogen balance) which reaches its maximum on the day before the end of the period of mania. The nature of the nitrogenous substances retained or excreted in this periodic way is not yet known.

Reviews on the following subjects have appeared: protein metabolism of malignant tissues, Voegtlin (133); nitrogen metabolism of plants, Vickery, Pucher, Wakeman & Leavenworth (41); metabolism of amino acids, ammonia, and urea in liver diseases, Kirk (130).

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THE METABOLISM OF CREATINE AND CREATININE*

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ANALYSIS

Previous reports have contained no discussion of analytical methods, which until recently consisted almost exclusively of modifications of Folin's picrate process. Guanidine derivatives, other than creatine and creatinine, have recently assumed physiological importance; not all of these respond to the Folin procedure, and new methods for their estimation have had to be developed.

Among compounds which develop color with picrate are those containing the group $\text{—COCH}_2\text{—}$ (Zimmermann). Polyhydroxylic phenols, which also may interfere (Linneweh & Linneweh), may be extracted from urine after acid hydrolysis without attendant loss of creatinine (Stelzer).

Removal of chromogens may also be accomplished by adsorbents such as Lloyd's reagent. Treatment with strong acid increases the activity of silicates for bases so that creatine and creatinine may be quantitatively removed even from very dilute acid solutions. For adsorption of different substances, see Zechmeister & Chlcnoky; Koschara; Ackermann; Fuchs; Weber; Thomas & Akao. Creatine and creatinine are not adsorbed by aluminum oxide (Th. and A. Remy), permutite [Weber (2), Bodansky], or basic lead acetate, so that these substances can be employed for preliminary purification. Suspended particles of adsorbent which may disturb colorimetry and photometry (Fisher & Wilhelmi) can be removed by using alcoholic picrate solution and precipitation by barium chloride after the color has developed. Elution is carried out by treatment with bases, such as magnesia, baryta, or even alkaline picrates. The method has been adapted to the determination of minute amounts by Borsook, and Fisher & Wilhelmi. Since the position of the equilibrium between creatine and creatinine depends upon the hydrogen-ion concentration, temperature, and solvent (Cameron & Guthrie), the standard procedures should not be modified without vigorous control of all details (Ciaccio; Lieb & Zacherl; Beard, Boggess & Pizzolato).

* Received February 7, 1938.

No agreement exists as to the optimal conditions for the conversion of creatine into creatinine. Control of the processes by added amounts is not satisfactory as other chromogenic substances are always present. Reported creatine values are, therefore, subject to an indeterminate error and small differences are not significant. A daily output of about 50 mg. of "creatine" is generally considered the smallest determinable amount.

Independently and almost simultaneously, Behre & Benedict and Langley & Evans reported the brilliant garnet red color obtained on treating creatinine with alkaline 3,5-dinitrobenzoate. In contrast to picrate, this reagent is colorless, but requires careful purification. The reaction is more specific than that with picric acid, in that glycohydrazide gives a color one-seventh as intense as with picrate, and hydantoin even less. It is, however, highly sensitive to changes in alkali concentration and light. Glucose and creatine give no color; acetoacetic acid reacts only in high concentration of alkali and the color fades more rapidly than that obtained with picrate. The new method has not replaced the picric acid method (Baier & Walter) in routine analyses of biological materials, but has proved valuable in scientific investigations.

Miller & Dubos report a highly specific enzymatic analytical method. The resting forms of four species of bacteria isolated from soil rapidly decompose creatine and creatinine with formation of urea. The degradation does not go by way of sarcosine, but probably by way of methylguanidine. Substances closely related to creatinine are not attacked, even glycohydrazide is attacked only one twenty-fifth as rapidly. This procedure has already produced striking results (p. 217).

Müller has been unable to reproduce Lang's method of determining creatine with benzoylacetyl.

Since the last report, glycohydrazide has acquired importance. Since it is a guanidine compound which contains no disubstituted amino groups, it gives the color reaction of Sakaguchi; this has been adapted by Weber and by Andes & Myers for the determination of glycohydrazide in urine. Guanidine itself does not give a Sakaguchi reaction, but reacts with 1,2-naphthoquinone-4-sulphonic acid (Sullivan).

THE DISTRIBUTION OF CREATINE AND CREATININE

While the muscle of horses is richer in basic extractives than that of cattle, pigs, or fowl (Yoshimura *et al.*), the content of creatine

and creatinine is approximately the same as in pig muscle, while that of carnosine is almost twice as great. Most of the creatinine, as well as the methylguanidine, are probably present as artifacts [cf. creatone, *Ann. Rev. Biochem.*, **4**, 245 (1935)]. The amount of creatine found depends upon the time between the death of the animal and extraction (Smorodinzew & Nicolewa). It is maximal twelve to fourteen hours after death, and then decreases, more rapidly at 36° than at 4° C. Creatinephosphoric acid, which is more difficult to extract than creatine, seems to be more firmly bound in fresh muscle. The muscles of different teleost fishes (carp, cod, perch, and pike) contain about the same amounts of creatine and creatinephosphoric acid, but less than that of mammals (Palladin & Sigalova).

CREATINE CONTENT OF HEART

Human hearts obtained at autopsy are not uniform, and much material is necessary to obtain statistical results. Bodansky, Pilcher & Duff (2) found creatine values of 38 to 295 mg. per cent in 301 hearts. In general, the left ventricle contained more than the right, and this relation was independent of age, sex, creatine, and phosphorus content, as well as the cause of death. Gradual myocardial failure produces a stronger decrease in creatine content than sudden failure (thyroxin), and for this reason, pneumonia, severe pulmonary disease, and congenital heart disease result in particularly low values of creatine in the right ventricle. The right ventricle of the fetus contains more creatine than the left, and shortly after birth the two values become equal at a higher level. The normal adult ratio supervenes during the first year of life with a bilateral increase of creatine content. In hypertension, sclerosis, lues, myocardial damage, and heart failure, the creatine concentration is usually low, particularly so in the infarction of coronary occlusion [Herrmann, Decherd & Oliver (1, 2)]. Seecof, Linegar & Myers [cf. *Ann. Rev. Biochem.*, **4**, 243 (1935)], as well as Kindler, consider 400 mg. per cent creatine the saturation level of myocardium. Kindler considers this value also maximal for decompensated hearts. With improved cardiac function the creatine decreases simultaneously with better oxygen and carbohydrate supply. Creatine thus seems to function as a substitute for other phosphate carriers. Mangun & Myers suggest that creatinephosphoric acid occurs in heart muscle as a dipotassium salt. Herrmann & Decherd *et al.* produced anoxemia by myocardial damage in

dogs, rabbits, and rats; this was accompanied by various chemical changes which in turn resulted in a further decrease of heart action. The resynthesis of lactic acid and creatinephosphoric acid is inhibited, the latter especially by the disappearance of sodium ions, and this in turn results in further decrease in creatine. An accumulation of lactic acid in the muscle in excess of 70 mg. per cent is associated with fatigue; heart failure is, therefore, always accompanied by a disturbance of glycogen- and creatinephosphoric acid-metabolism. The bladder urine of cattle killed by exsanguination contains much more creatine than that of animals slaughtered by stunning. The creatinine content of both is the same. Feldmann ascribes this difference not to alteration of kidney function but to the inability of the active but asphyxiating muscle to retain its creatine as it would were there an ample oxygen supply.¹

The changes in the structure of the tissues, and varying amounts of edema fluid with a creatine content near to that of blood plasma, influence the value. These individual factors are particularly noticeable in surviving hearts. Herrmann, Decherd & Ehrhard found in rabbit hearts which had been perfused for only one minute, 153 mg. per cent of creatine in the fresh muscle and 795 mg. in the dried tissue. If, however, they perfused for one to five hours with Ringer-Locke solution with a content of 0.1 per cent glucose and oxygen, they found on the average only 123 mg. per cent (742 mg. on dry weight basis). This loss was obviously due to edema. More precise analyses are necessary. A careful repetition of this work by Fisher & Wilhelmi showed that the creatine content of rabbit heart is dependent on the size and age of the animal. It appears that before puberty, heart weight and creatine synthesis increase simultaneously. During further development of the heart, creatine synthesis is inhibited in some way and the creatine content does not reach its optimal level.

CREATINE TOLERANCE

The work of Benedict & Osterberg has shown that, on administration of large amounts of creatine for long periods, the creatine content of the tissues rises and increased excretion of both creatine

¹ Phosphate and creatine diffuse from frog muscle into Ringer's solution more slowly in the fatigue state (Cardoso).

and creatinine results. This is not observed with small doses. Administration of 75 mg. daily for four to six months to young and adult rats does not increase the values in gastrocnemius, heart, and liver (Struck & Vischer). In a child suffering from myotonia congenita, whose condition had improved by treatment with thyroid, resulting in loss of creatine, the muscular tone increased on administration of creatine or by raising the body creatine in other ways (Poncher & Woodward). The administration of 50 to 100 mg. of creatine to rats results in a small and transient increase of the creatine content in the striated muscle but not in heart muscle. Irregular and temporary increases were found in the liver and kidney.

Determination of creatine tolerance has become a routine clinical procedure. Alimentary creatinuria occurs when the capacity of the tissues and of the blood for degradation and storage no longer suffices and if the kidney threshold is exceeded. The test thus comprises three factors of unknown magnitude. Nitzescu & Gontzea have found that on administration of increasing quantities of creatine to humans by mouth, creatinuria appeared in 20 per cent of normal humans only on feeding 1.5 gm. or more; with 2 gm. 60 per cent of the cases excreted 5 to 15 per cent in the urine. On the other hand, when administered intravenously, as little as 0.5 gm. was incompletely retained by 20 per cent. The rate of injection seems to be involved. Creatinuria is more prevalent in the test in cases of liver disease, so it appears as if the liver is the principal site of degradation of the creatine administered. When this function of the liver is impaired, with resultant creatinuria, a reduced storage capacity of the other tissues is indicated. This hypothesis would explain the constancy of the creatine level on the assumption that creatine is normally produced at a constant rate. Richardson & Shorr administered 1.32 gm. of anhydrous creatine to adults by mouth and regarded those cases as normal in which a spontaneous creatinuria of 50 to 60 mg. per day resulted and less than 70 per cent of the ingested creatine was retained. Marples & Levine, as well as Kleinschmidt and others, regard 30 to 60 mg. per kg. as the lower limit. It seems preferable that all investigators should determine standard values for themselves. It must be borne in mind that in creatine-tolerance tests only 1 to 2 per cent of the amount stored in the body is given, while in sugar-tolerance tests about 100 per cent is given. On simultaneous addition of glycine, creatine is not as well tolerated by normal subjects as by patients with muscle disease (Adams, Power & Boothby; and

Milhorat & Wolff). The tolerance is reduced on administration of thyroxin or of pituitrin (Acromegale S.).

THE CREATININE OF THE BLOOD

The question of whether creatinine is present in the blood has been discussed in earlier volumes [*Ann. Rev. Biochem.*, 2, 187 (1933); 4, 244 (1935)]. The answer depends upon the possibility of determining traces of creatinine in the presence of relatively large amounts of creatine in dilute solution without converting the compounds into each other. The progress in analytical methods has been discussed in the first section. Linneweh, employing Lloyd's reagent, was able to isolate very small quantities of creatinine. Zacherl supplemented the colorimetric method (Zacherl-Lieb) by preparative procedures. Creatine was separated from creatinine by precipitation with mercuric acetate. The resulting creatine must have been present as such in the blood. Ferro-Luzzi precipitated the blood proteins with zinc hydroxide and found lower Jaffe values than in Folin-Wu filtrates; these values are probably closer approximations to the truth. Hayman, Johnson & Bender, when employing Lloyd's reagent, obtained so consistent results for creatine and chromogen as to lead them to conclude that creatinine or a closely related derivative of it is present in plasma.

Danielson, in comparing the chromogen of ultrafiltrates and the filtrates from tungstic acid precipitations with the behavior of creatinine solutions under different conditions, concluded that the greater part of the chromogenic material in normal plasma is creatinine. However, Behre & Benedict (3) were unable to confirm this view; their new reagent, *m*-dinitrobenzoic acid, is more specific for creatinine and the colors obtained from blood and plasma filtrates did not conform with those from pure creatinine either in tint or in rate of development. With suitable modification of the dinitrobenzoic acid technique, however, no marked differences were observed (Bolliger; Langley & Evans; Zacherl). Attempts to precipitate the chromogen from blood filtrates by means of rubidium picrate (to which creatinine readily responds) were unsuccessful (Behre & Benedict). Gaebler, however, reports that in ultrafiltrates of normal human serum the chromogen is slowly but incompletely precipitated by this reagent; his results indicate that normal human serum contains creatinine, but in amounts less than 1 mg. per cent, a value also found by Popper,

Mandel & Mayer. Creatinine could not be detected in dog and cattle serum by means of rubidium picrate, although added creatinine could be quantitatively recovered.

Miller & Dubos have furnished an indirect proof of the presence of creatinine in serum. On treating blood, serum, plasma, erythrocytes, and spinal fluid with their strains of bacteria in resting form (see p. 212), chromogen disappeared in amounts equal to those indicated by the picrate and dinitrobenzoate methods. This agreement excludes the presence of glycohydrazide; and since the chromogen of spinal fluid, which is a natural ultrafiltrate of plasma, is completely destroyed by the bacteria, these experiments taken together point strongly to the presence of creatinine in serum.

Recent work thus makes the existence of creatinine in plasma highly probable. This simplifies the question of the origin of urinary creatinine. In 1930, Van Slyke, Hiller & Miller found the excretion threshold of creatinine added to plasma to be 20 mg. per cent for plasma and 11 mg. per cent for whole blood. If these values apply for natural chromogen, the blood in the renal vein should contain 0.11 mg. per cent less than that in the artery. Goudsmit, in thirty experiments on dogs, found the extraction percentage to lie between the values 7.3 and 16.8 per cent, averaging 11 per cent. The natural chromogen thus has the same excretion ratio as creatinine, in good agreement with the results of Miller & Dubos, who by their indirect method recognized 91 per cent of the chromogen as creatinine. Similar experiments by Zacherl indicate that oxalated blood or serum loses part of its chromogen on passing through the kidney. Thus this organ is not the site of formation of urinary creatinine; this must be sought in other regions from which it is transported by the blood to the kidney.

Before the presence of creatinine in plasma had been definitely established, the possibility of its exclusive formation in the kidney had always to be considered. This involved the unlikely assumption that the increase of serum creatinine in cases of renal insufficiency was due to a reabsorption from the kidney into the blood stream. The impaired kidney would thus have to be more efficient in this respect than the normal organ, even if the blood supply were restricted. Bolliger & Carroddus, working with dogs, transplanted one kidney to the omentum, and after it had attained a new supply of blood vessels, the original blood vessels were ligated. During the resulting acute uremia the plasma creatine and creatinine, determined by

picrate and by dinitrobenzoate, increased. Creatinine was distributed equally between plasma and corpuscles, whereas creatine remained chiefly in the plasma. Even when both kidneys were removed, the blood creatine rose from 2.57 to 17 mg. per cent, and the creatinine from 1.4 to 8.4 (Scaffidi & Giliberti). Proof is thus furnished that the kidney is not the sole site of creatinine formation, which probably takes place in the muscles. In a case of congenital muscular hypertrophy unusually large amounts of creatinine were excreted, together with creatine, which was badly tolerated. Potassium, phosphorus, and phosphatase were above normal (Hall, Sundermann & Gittings).

ORIGIN OF CREATINE

The question of whether arginine is the precursor of creatine is still unanswered, but it seems probable that this substance is associated with creatine formation. Perfusion experiments by Hongo with the stomach and Herrmann & Decherd with the heart have been superseded by the work of Fisher & Wilhelmi. Experiments with the intact animal are not clear-cut, as an increased excretion may be dependent on liberation of stored material, on metabolic changes, or on renal activity. Hypothetical precursors may follow some other degradative path, so that the quantitative conversion into creatine, necessary for proof, cannot be expected. The isolated rabbit heart can be maintained intact in a closed circulatory system, and its activity followed by its rhythm. About 100 hearts were perfused with Ringer-Locke solution containing 0.2 per cent glucose for two hours without change in rhythm. Difficulty was encountered in setting normal standards, as the age, size, and dry weight of the heart had to be considered. In the hearts of immature animals of body weight less than 1800 gm., the creatine level increased more rapidly than with older animals. Glucose perfusion alone had no effect on creatine content, which depended upon the size of the heart, i.e., the age of the animal. On the addition of a hypothetical creatine precursor the increase in creatine had to be greater than the significant differences of the control values. In 25 experiments the addition of arginine led to an indubitable increase. With adult hearts, which do not contain the optimal amount of creatine, arginine disappeared with an equivalent increase of creatine. As guanidinoacetic acid and creatinine were not formed, the results offer definite proof of the conversion of arginine into creatine. The failure of similar experiments

previously carried out on the whole animal is to be ascribed to the fact that only insufficient amounts of arginine could be supplied, and that arginine is degraded in other ways. When large quantities of arginine were supplied, an increased creatine excretion pointed at least to this relation. Earlier perfusion experiments suffered from lack of statistical interpretation and of the adequate consideration of the apparent increase in weight due to edema, the insufficient addition of glucose, and the differences between immature and adult hearts.

The amount of creatine formed does not depend solely on the amount of precursor employed, as other limiting factors are involved. In contrast to earlier findings, arginase is shown always to be present in voluntary muscles. No definite relationship between creatine, arginine, and arginase was observed in fowls and rabbits (Palladin & Rashba).

Guanidinoacetic acid is the only compound which is excreted to a considerable extent in the form of creatine. After its presence in the urine in cases of muscular dystrophy had been demonstrated by Weber [cf. *Ann. Rev. Biochem.*, 4, 252 (1935)], it was found in the urine of normal humans, dogs, and rats (Weber; Ackerman; Bodansky, Duff & Herrmann; Sullivan). The quantitative determination in the urine of humans (20 to 40 mg. per day) and rats (0.5 mg. per day) has been carried out only colorimetrically. Isolation procedures, which involve losses, have led to its identification in the urine of dog and man. A notable increase of urinary creatine follows the administration of guanidinoacetic acid only when the creatine tolerance is small, as in cases of muscular dystrophy. Guanidinoacetic acid has not been detected in muscle, heart, or liver of the rat, but traces may be present in kidney and intestinal tract. When fed, 50 per cent soon appears in the urine, and its presence can be detected in liver and kidney, but not in muscle and heart. Bodansky regards the methylation as taking place in the kidney but does not exclude muscle, which may under normal conditions be the principal site of this process. It does not occur in the liver, for Bodansky found distinct evidence of deposition of guanidinoacetic acid in this organ, without increase in creatine. This fails to concord with the claim of Zappacosta that intravenous injection of guanidinoacetic acid forms a sensitive and specific liver-function test, based on the rapid disappearance from normal plasma of the Sakaguchi reaction, which persists in liver disease. Apparently the tissues of normal humans take up glycoamine more readily, and this tolerance is lowered in liver disease [Nitzescu & Gontzea (2)].

Guanidinoacetic acid cannot yet be regarded as the immediate precursor of creatine. Proof of its presence in normal muscle is still lacking, and Fisher & Wilhelmi were unable to find it in perfused hearts. However, administration of glycine leads to increased excretion not only of creatine but of guanidinoacetic acid in cases of human muscular dystrophy (Weber) and in rats (Bodansky). This guanidinoacetic acid is not necessarily related to creatine formation in muscle.

Glycine is now frequently employed therapeutically for muscular dystrophy and attempts have been made to influence other diseases or symptoms such as fatigue, muscular weakness (even that in Addison's disease), anorexia, and emaciation (Beard). This is not the place to review such clinical investigations nor to interpret the nature of these diseases from their reactions towards glycine. Reference may be found in the papers of Borst & Möbius; Braestrup; Espersen & Thomsen; Boothby; Adams, Power & Boothby; the papers contain critical reviews of the literature. Systematic studies of the total intermediary metabolism of muscle in the interesting nutritional muscular dystrophy of Goettsch and Pappenheimer have of course included the relations of creatine and creatinine in this condition. These studies, however, have contributed but little to the solution of the physiological problems, for the dystrophic state sets in very suddenly after a long and uneventful prodromal period. Although it has been impossible as yet to attain a uniform condition of long duration, such as would be necessary for metabolic balance experiments, the experimental production of this disease has proved fruitful in other investigations (Morgulis & Spencer; Ni).

Creatinuria following administration of glycine to dystrophic patients is always greater than with normal controls, and is too great to be accounted for as guanidinoacetic acid. Extra creatine persists in the urine for varying periods, but may decrease even during the administration of glycine. Adams, Power & Boothby, observing more than 100 persons for periods up to more than a year, noted that the majority (healthy and myasthenic) excreted little or no creatine, in contrast to patients with muscular dystrophy. Administration of 10 to 30 gm. of glycine almost always resulted in the establishment or the increase of creatinuria. This action of glycine takes effect after widely different lengths of time, in some cases on the first day, in others after several weeks. Once established, creatinuria increases steadily so long as glycine is given. No definite relation could be estab-

lished between the amount of glycine administered and the quantity of creatine excreted; the latter was always very slight. The findings may be taken, perhaps, as indicating merely an indirect stimulation of metabolism rather than a direct conversion. Creatine tolerance decreases on administration of glycine, but the increased creatinuria cannot be ascribed to a temporary displacement of tissue creatine by glycine.

A second criterion for increased creatine formation is the creatine content of the tissues. It is hardly influenced by feeding creatine itself (cf. Bodansky). It is not surprising that feeding 100 to 200 mg. of guanidinoacetic acid, or 1 gm. of glycine causes no appreciable increase in muscle creatine; on feeding 2 gm. of glycine, 0.5 gm. of extra guanidinoacetic acid was excreted in the urine, but none was found in liver, heart, or muscle. Glycine does not increase the creatine content of voluntary muscle or heart muscle which had previously been depleted by experimental hyperthyroidism (Bodansky & Duff).

Administration of fifty-gram doses of glycine to normal adults has no effect on urinary creatine and creatinine (Borst & Möbius); doses of 25 gm. are normally deaminated to the extent of 48 per cent and excreted as urea in one to three hours, but more slowly in nephritis (Kirk). Daily doses of 4 gm. are toxic to fowls after a few days; egg production ceases, extreme muscular weakness supervenes, and sudden clonic spasms occur, but all these symptoms vanish at once on discontinuing the administration of glycine (unpublished observation by the reviewer; cf. Patton & Palmer).

Glycine is utilized for hippuric acid synthesis by children (Marples & Levine) and by dystrophics to the same extent as by healthy adults, and therefore must be present in sufficient amounts in those organs in which this synthesis occurs [Linneweh & Linneweh (1); Thomsen]. It is also formed in sufficient amounts in normal animals, as is indicated by the growth experiments of McCoy & Rose. It is also present in the muscles of patients with muscular and nervous diseases, as in the dystrophic muscle the collagen content is even several times greater than normal (Spencer, Morgulis & Wilder). However, it may not be supplied to the sites of creatine formation, for only in certain types of muscular disease does glycine lead to a greater creatine excretion than in healthy individuals. Equimolecular amounts of glycolic acid have a smaller effect in the dystrophic patient (Milhorat & Toscani).

Glutamic acid, tested on normal and myasthenic patients (Adams,

Power & Boothby) and on toxic peripheral neuritis (Allinson, Henstell & Himwich), and arginine, in the form of edestin, do not have this effect. They fail to improve the clinical condition of dystrophics, as is sometimes the case with glycine. Beard and collaborators, however, maintain their original position [cf. *Ann. Rev. Biochem.*, 4, 248, 251 (1935)] and have extended their studies on a broader basis, employing rats.

RELATIONS BETWEEN EXCRETION OF CREATINE AND CREATININE, AND OTHER FACTORS

Creatinuria and disturbances of sugar metabolism.—It has long been known that creatinuria is associated with lack of available carbohydrate, which is not surprising in consideration of the close relation between phosphocreatine and glycolysis. In Volume IV the postulated relations are discussed in connection with the papers of Brentano, Riesser, and others. Since then no new principles appear to have emerged. It is not known how creatine is reabsorbed from the glomerular filtrate nor how the muscle protects itself against loss of creatine during its transient liberation. It is impossible, therefore, by reason of the small deviations of the amounts of creatine excreted, to estimate the extent to which muscle metabolism contributes to creatine formation and the extent to which renal function has failed.

Creatinuria and proteins.—Rose concluded his report [*Ann. Rev. Biochem.*, 4, 258 (1935)] on the paper from Terroine's laboratory: "The reviewer is not yet convinced that the production of creatine can be augmented from exogenous sources beyond the physiological needs of the organism for it." In reference to this opinion, Terroine with Bonnet & Maurat has again stated in detail his view that endogenous and exogenous protein metabolism influences creatine excretion; as both processes are closely related quantitatively, their connection is not subject to question, although the mechanism is still obscure. Synthesis from tissue material is definitely established in, for example, complete or specific protein deficiency. At Terroine's instance, Mourot has confirmed previous determinations of its extent. It seems to the reviewer that Terroine's interpretation has not yet met with general acceptance. The creatine content of hearts which have been rendered hypertrophic or otherwise damaged was found to be independent of the amount of creatine or of protein supplied with the diet, as is the case with creatinuria in children (Chanutin & Ludewig).

The next few years may show whether the findings of Beard will form the desired bridge to the solution of the problem.

Metabolism of energy.—Endogenous nitrogen metabolism holds a definite relation to energy metabolism. As the latter increases by muscular activity in specific nitrogen starvation, nitrogen excretion increases proportionally (Metzincesco); creatinine excretion, however, remains constant, while creatine values yield no information. On increasing the energy output for thermal regulation, a confusing picture of creatine-creatinine relations results. Terroine, Bernardie & Lelu held adult rats in thermal equilibrium at 30° and at 5 to 9°. At the lower temperature, creatinine excretion rose by about 30 per cent, the slight creatinuria quadrupled in three out of six animals, while the urinary volume decreased markedly. Creatine tolerance fell: Injection of 2 mg., which was well tolerated at 30°, brought about an extra excretion at 10°. When the metabolic rate was raised by chemical agents such as dinitrophenol, excretion of both creatine and creatinine rose unexpectedly (Pugsley). Creatine excretion rises with the resulting fever, and the tolerance falls from a normal value of 80 to 100 per cent to 25 to 50 per cent (Lieben & Asriel).

Thyroid.—Hyperthyroidism lowers the creatine content of tissues and muscle (particularly heart muscle), resulting in creatinuria (Bodansky). The circulation is regularly impaired when the creatine content of heart muscle decreases from 190 to 90 mg. per cent (Bodansky & Pilcher). One mg. of thyroxin damages adult rats more than immature or pregnant animals. Normal growth is retarded. The heart hypertrophies and loses creatine, but functions better than adult hearts having the same creatine content. On discontinuing the thyroxin, the ventricle regains its normal creatine content in seven to fourteen days, and while the hypertrophy subsides more slowly, normal growth is soon resumed. This corresponds to the small changes in fetal hearts as compared to those of mothers receiving thyroxin (Bodansky, Duff, Herrmann & Campbell). An external temperature below 20° leads to hyperplasia of the thyroid; this does not occur if thyroid or thyroxin be given. The thyroid does not store colloid, but the creatine content of the myocardium remains high (Bodansky & Pilcher, Bodansky & Duff). When rats damaged by thyroxin and having low myocardial creatine receive much sugar and so replenish the glycogen reserve of the heart, the creatine falls less than when creatine alone is fed, for the latter is not well stored under the influence of thyroxin. A good example is the case of a child with

myotonia congenita who showed no creatinuria on a high protein diet but developed it on thyroid medication (Poncher & Woodward).

Treatment with iodine relieves hyperthyroidism, diminishes creatinuria, and increases creatine tolerance, but does not influence creatinine excretion (Rödland & Wang). The thyrotropic pituitary hormone also induces creatinuria (Pugsley, Anderson & Collip), which is not reduced by the antithyrotropic substance. The growth and adrenotropic hormones of the pituitary do not cause creatinuria.

Gonads and hypophysis.—In last year's *Review* the difference in metabolism between humans and rabbits was discussed. New experiments on humans (Nitzescu & Gontzea) have confirmed earlier reports. Sex hormones decrease physiological creatinuria and raise creatine tolerance. Prolan, by inducing gonad activity, induced similar effects in an achondroplastic and infantile dwarf. Anterior pituitary hormone has the same effect. Prolan diminishes creatinine excretion, and "Enarmon" reduces creatinuria in old men and Addisonian patients (Usui, Miwa & Aoki). The influence of the anterior pituitary is evident from observations on nine acromegalic patients and from injection of the hormones into normal men (Shire). After intramuscular injection of antuitrin G, creatinine excretion rose; creatine was absent from the urine. On injecting thyrotropic hormone, 0.65 gm. of creatinine was excreted per day, while creatinine remained constant. Acromegalics excrete much endogenous creatine and creatinine, in amounts which may vary from day to day. The secretion of eosinophilic tumors seems to influence the muscle so as to produce more creatinine, while the thyrotropic hormone induces an increased permeability of the kidney for creatine. The pronounced variations in excretion may thus be ascribed to varying activity of these hormonal secretions of the tumor.

Shapiro & Zwarenstein have transferred their investigations on the influence of pituitary from the rabbit to the South African toad *Xenopus laevis*, from which the gland can readily be removed. Several hundred experiments, treated statistically, indicate that hypophysectomy leads to slow loss of muscle creatine, which appears after six to ten and becomes pronounced after eighteen to twenty-two weeks. This may be ascribed to the gradual inactivation of the gonads. Repeated small doses of anterior pituitary extract lead to a higher creatine content than one massive dose. The experiments were well controlled by observing the effects of captivity, injury to other portions of the brain, removal of either the anterior or the posterior lobe,

and injection of posterior pituitary extract. The influence of the growth hormone has been studied by Bartoli, Reed & Struck. Complete or partial removal of the pituitary induces a transient creatinuria (Perla & Sandberg).

Creatinuria in children.—In about five out of seven children observed by Kleinschmidt the creatinuria disappeared on a diet low in protein, but reappeared when 5 gm. of glycine was given daily. It would be interesting to ascertain whether the creatine values are ascribable to the presence of guanidinoacetic acid. Low protein diets did not have the marked effect on children with Erb's dystrophy, but increased creatinuria resulted from addition of glycine. Thus determination of creatine tolerance has no diagnostic value in children. Marples & Levine, working with eight healthy children aged from three weeks to seven months, found the total creatine coefficient to have the same order of magnitude as the preformed creatine coefficient of adults. The lowest values and greatest variations were found in children under one month old. Creatinuria was absent in premature infants and in some newly born at term. Adequate, but protein-low diets, decreased, and high protein diets increased, creatine values. Children tolerate creatine poorly; when 35 to 60 mg. per kg. were given, 55 to 65 per cent of ingested creatine was excreted on the first day, and 63 to 82 per cent had appeared after two days. Glycine increased the excretion of creatine, but not of creatinine. No quantitative relation was found between creatine excreted and glycine fed. Catherwood & Stearns determined the creatine and creatinine excreted by 23 new-born infants. At birth, 4.6 mg. per kg. were excreted; the amount rose slowly to 10.5 mg. per kg. at fifteen to twenty weeks, and then remained constant. The quantity depends essentially upon the amount of muscle, as shown by the statistical relations between body weight, height, and age. Children who had received milk enriched with protein from the tenth to the fourteenth day, until their weight had tripled, excreted somewhat more (10 to 14.5 mg. per kg.), from which it may be concluded that muscle growth was more rapid than with children who received only breast milk. The level of creatinuria possibly depends on thyroid function, for the curves for creatine (mg. per kg.) run parallel with those for energy output (kilocal. per kg.) with increase of age. The creatinine excretion equals or exceeds that of creatine; the amounts of the two change in neither direct nor inverse ratio, so that they appear to express two independent phases of muscle metabolism (cf. Poncher & Woodward

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ACID-BASE METABOLISM*

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The multiplicity and diversity, not only of publications, but of subjects and even of entire fields, which may properly be discussed under the above title make impossible any adequate review within the space allotted for this chapter. Since the topic has not previously appeared in this series of reviews, the writer's task would seem to be even more difficult. Fortunately, the splendid and exhaustive compendium of clinical chemistry by Peters & Van Slyke (1, 2), who have adequately presented the status of the subject in its many aspects up to the period ending in 1931, makes necessary only scant reference to work prior to that time. Furthermore, much work done since has been merely a repetition of earlier, well-known, observations. Mention of most of this material has been omitted. Embracing a more limited and slightly different territory from that covered with Van Slyke, Peters (3) has summarized, somewhat more speculatively, but nevertheless stimulatingly and adequately, certain pertinent additions to our knowledge up to the beginning of 1935. With the aid of various other relevant reviews the writer has endeavored to bring up to date certain recent trends, and to enumerate points of importance and interest. The discussion has been quite arbitrarily limited mainly to a consideration of some of the developments in acid-base balance studies of blood and urine.

Acid-base metabolism, strictly speaking, would be concerned with the various amounts and kinds of acids and bases taken in by the body, or produced and eliminated in metabolic processes. In this review, however, attention will be confined rather to one phase of the mechanical and chemical system operating to maintain physiological neutrality under variable metabolic conditions. As descriptive of the chemical state of the body in this respect, Van Slyke has suggested the term *acid-base balance*, now more generally used in connection with studies of blood, the pH and alkaline reserve of which have proved to be a reliable index of acid-base conditions in other tissues.¹

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¹ In this chapter we shall refer to "blood" without differentiating between its constituents, plasma and cells. When necessary to distinguish them from

Standardization of pH measurements.—The focal point of interest in acid-base balance measurements is the pH. The development of new, more convenient techniques, particularly those involving the use of the glass electrode, have led to the recording of values of pH as unqualified as ordinary observations of temperature. The development of comparatively easy pH measurements is not an unmixed blessing. It is tremendously advantageous and quite satisfactory for most physiological purposes. It affords measurements of pH under some previously impossible conditions. However, the tendency to avoid any mention of the basis of standardization has served to make the validity of the results obscure in many cases. On the other hand, the theoretical basis upon which pH values were thought to rest has become less certain, and the problem of standardization correspondingly more difficult.

In the early work on the essential constants necessary for the description of blood as a physicochemical system, it was realized that hydrogen-electrode measurements did not yield hydrogen-ion concentration values. The concept of activity offered greater theoretical support for what were then assumed to be measurements of hydrogen-ion activity (4, 5, 6, 7). Clark (8) soon called attention to the existence of such uncertainties with regard to liquid-junction potentials and the hydrogen-ion activity of any standard solution, as to make desirable the adoption of an arbitrary but definite meaning for pH values. More recently, MacInnes (9) has again pointed out that the potentials of the hydrogen electrode cannot be interpreted as a measure of hydrogen-ion concentration. He has shown further that since neither individual ion-activity coefficients nor single liquid-junction potentials can be measured, hydrogen-ion activity cannot be defined thermodynamically. He has recommended the retention of the usefulness of the term pH, as related to potentials of the hydrogen electrode, through a scale based on thermodynamic dissociation constants.²

each other, plasma or serum, cells, and whole blood will be mentioned specifically. With regard to pH of serum and of whole blood, the distinction is merely one of method, indicating the material directly used for measurement. It is now accepted that the pH of blood is the pH of the plasma in which the cells are suspended.

² MacInnes & Longworth (13) eschew the use of the terms "hydrogen-ion concentration" and "hydrogen-ion activity," preferring simply pH. Clark (14) now proposes to abolish that term also, and to substitute the E.M.F. values from which they are derived.

Following the suggestions of Clark (8) and MacInnes (9), Hitchcock (10, 11) has established a satisfactory pH scale based on the known values of the thermodynamic dissociation constants of a number of weak acids. His pH values for several standard solutions do not vary significantly from those previously used by workers who had attempted to define pH on an activity basis. In this reviewer's opinion, the results furnish an additional argument for the abandonment of the now prevalent 0.1N HCl, and the use of some buffer mixture such as that of acetates, as the ultimate standard (12).

METHODS OF pH MEASUREMENT

Hydrogen electrode.—Despite great activity in the investigation of pH measurements by methods other than that of the hydrogen electrode, the latter still remains the classical standard of reference, upon which the others depend. The only recent development in hydrogen electrodes has been the rotating electrode of Du Nouy (15, 16, 17, 18) for which an accuracy of 0.01 pH is claimed. Only 0.4 cc. of plasma sample is required, and equilibrium is reached more rapidly than with the rocking type of electrode. No extensive use has been made of this method, which has much in its favor and should be more rigorously tested. Strangely enough, one still finds attempts being made to use the hydrogen electrode for measuring the pH of oxygenated whole blood (19, 20).

Quinhydrone electrode.—Because it is subject to various sources of error (salt and protein effects, drift of potential, and variation in reproducibility), the quinhydrone electrode is not suitable for accurate pH measurements of biological fluids. No significant improvement in such applications has been made since the work of Cullen & Earle (21). Laug (22) and Yoshimura (23) have confirmed the essentially empirical character of the results. Under the best of conditions the accuracy for serum is probably 0.03 pH.

Colorimetric determination of pH.—One would expect that the colorimetric determination of pH would share in the benefits of the increasing use of photometers, visual and photoelectric, and, indeed, steps have been taken in this direction. However, the accuracy of any colorimetric method is limited by the reactions governing the development of the color. The colorimetric measurement of serum pH is a good example.

As developed by Cullen (24), the colorimetric readings at 20° were related to hydrogen-electrode measurements at 38°, by a correction constant, *C*. Hastings & Sendroy (25) subsequently made

colorimetric readings at 38°, finding little variation from the electro-metric results at that temperature. Further work revealed variations, from time to time, in the *C* correction, and necessitated an *H* correction (26) for the Hastings & Sendroy method. On the whole, results by either technique have been found satisfactory for most clinical work.³ However, the discrepancies mentioned have occurred often enough under apparently abnormal conditions to make them the subject of much study in the laboratories of Cullen (27, 28) and of Myers (29, 30). Robinson, Price & Cullen (28) have recently found the *C* correction to vary directly with the logarithm of the protein concentration of the serum. Occasionally, they report, other factors may have even greater effect than has change of protein, on the magnitude of the *C* correction. Their results satisfactorily shed light on what has been a perplexing problem. Since control of the responsible factors, even if they were all known, would be impracticable in this reviewer's opinion, these results indicate definitely that colorimetric serum pH measurement must be limited to work requiring an accuracy no greater than 0.05 pH.

Glass electrode.—Since 1925, when Kerridge (31) called attention to the use of the glass electrode for pH measurements of biological fluids, considerable progress has been made in the development of various types of apparatus particularly designed for such work. There are many variations and modifications in the literature, based mainly on work of MacInnes & Dole (32, 33), Stadie (34), Hill (35), and MacInnes & Belcher (36, 37, 38). The present status of the glass electrode has been more recently outlined by MacInnes & Longworth (13).

Although theoretical support for its action is still obscure, it has been shown that the potential of the glass electrode in the usual galvanic cell system is quantitatively related to change in pH, as is the potential of the hydrogen electrode. Because it is unaffected by the action of substances which interfere with pH measurement by other methods (mixed oxidation-reduction potentials, oxygen, protein, etc.) it has come into widespread use in the biological sciences. The results are empirical, and must be referred to the hydrogen electrode

³ Since the major part of the *C* correction is a result of the difference in temperature between 20° and 38°, the Hastings & Sendroy technique should have a slight advantage in accuracy, since there is no reason to expect the temperature coefficient of the colorimetric readings to be constant from one sample to another.

by standardization (preferably with two standards at widely separated pH values). Where accuracy is concerned most of the values in the literature should be regarded with a great deal of skepticism because they lack the support of such reference values. Further confusion has been created by the lack of distinction between "sensitivity" and "accuracy." An instrument sensitive to changes of 0.01 pH may yet show an agreement of only 0.1 pH with the hydrogen-electrode value for a given solution.

Stadie, O'Brien & Laug (39) have made the best comparison of glass- and hydrogen-electrode results for serum, obtaining agreement within 0.01 pH. Sendroy, Shedlovsky & Belcher (12) have compared results for reduced whole blood, obtaining a similar agreement. From this, one would expect the glass electrode to yield results of the same accuracy for the pH of oxygenated whole blood, which can be measured directly by no other method. Another task for which the glass electrode is well suited is that of automatic pH control, for which apparatus have been described by Longsworth & MacInnes (40), Muller & Durichen (41), and Schwabe (42).

DETERMINATION OF ACID-BASE BALANCE CONSTANTS OF BODY FLUIDS

Values of pH are important, not in themselves, but because they indicate ratios of dissociated anions to undissociated acid, according to the familiar Henderson-Hasselbalch form of the buffer equation

$$\text{pH} = \text{pK}' + \log \frac{[\text{BA}]}{[\text{HA}]} \quad (\text{I})$$

which has been used for blood and body fluids in the form

$$\text{pH} = \text{pK}' + \log \frac{[\text{B}\ddot{\text{H}}\text{CO}_3]}{[\text{H}_2\text{CO}_3]} \quad (\text{II})$$

where pK' is, so to speak, the "keystone" constant under a given set of conditions. Peters & Van Slyke (1) have discussed work done on the evaluation of constants (up to 1930) and the application of Equation II in detail. Developments since that time are outlined in the following.

MacInnes & Belcher (43), by electromotive force measurements on galvanic cells without liquid junctions, and Shedlovsky & MacInnes (44), by conductance measurements, have confirmed the earlier values of Hastings & Sendroy (4) for the first and second dissociation constants of carbonic acid in salt solution at 38° . The disturbing

disagreement (7) between different laboratories as to the value of pK' in serum no longer exists. Robinson, Price & Cullen (45) have very carefully and accurately repeated measurements of the pK' of 138 dog and human sera. Their average value of 6.095, and the pK' of 6.105 found for human, ox, and dog sera by Dill, Daly & Forbes (46),⁴ agree excellently with Hastings, Sendroy & Van Slyke's (7) value of 6.105.

Dill, Daly & Forbes (46), using a glass electrode, have also repeated the evaluation of pK' for human red blood cells at 37°. Making some necessary recalculations in older data to conform with work done since, they find that their average values of 5.98 and 6.04 for reduced and oxygenated cells, respectively, compare well with those of previous workers. Thus, they find revised values of 5.93 to 6.07 and 6.10 to 6.12 for reduced and carbon-monoxide saturated (the equivalent of oxygenated) hemoglobin solutions, respectively (47), and 5.97 for reduced horse cells (5).⁵ The discrepancy between serum and cell (or hemoglobin solution) pK' , the dependence of the latter pK' on pH, on the hemoglobin concentration (at constant ionic strength) and its degree of oxygen saturation, are now beautifully accounted for by the revelation that pK' for solutions containing hemoglobin is not a true constant, because the bound carbon dioxide is not all in the form of bicarbonate, but also includes a portion bound to hemoglobin as carbhemoglobin, a carbamate (48, 49, 50, 51). However, although theoretically incorrect, the use of pK' for hemoglobin-containing solutions loses none of its conveniently practical advantages in the description of acid-base phenomena where it is not necessary to distinguish between, or separate, the component parts of the total bound carbon dioxide.

The same may be said of pK' for whole blood which is nothing but a hybrid "constant" for a heterogeneous system of cells in plasma. The values are affected not only by the factors affecting cell pK' mentioned above, but also by the inequalities in the distribution of carbon dioxide between cells and serum and the preponderance in the cells of the carbamino compound, of which there is but little in the plasma (51).

⁴ Dill, Daly & Forbes actually found pK' to be 6.11 at 37°. Their reported temperature coefficient of -0.005 for every 1° C. has been used by the writer to compare their values with the others found at 38°.

⁵ For 38°, all of these pK' values for cells should be less than 0.01 unit lower; see footnote 4.

In the light of later work, Van Slyke & Sendroy (52) have made a more accurate evaluation of pK' for whole blood as related to pK' of serum (53, 5), and have indicated its practical usefulness in empirical acid-base balance relationships for whole blood and serum.

Employing the same methods used for blood work, Sendroy, Seelig & Van Slyke (54) have accurately redetermined the values of pK' and α (the Bunsen solubility-coefficient of carbon dioxide) for human urine at 38°. The variations found (pK' , 5.97 to 6.21, average = 6.10; α , 0.498 to 0.536, average = 0.522) fluctuated within narrower limits than the results of Mainzer & Bruhn (55), and were chiefly a function of the salt concentration.

GRAPHIC CALCULATION OF ACID-BASE BALANCE DATA OF BODY FLUIDS

Nomograms and charts designed to facilitate the calculation of acid-base relationships, to describe the course or path of the changes involved, and to interpret or classify them, continue to appear from time to time. For the application of the Henderson-Hasselbalch equation to whole blood analyses, Van Slyke & Sendroy (52) have revised the nomogram for the evaluation of pK' of whole blood as affected by the factors pH, hemoglobin concentration, and oxygen saturation (5). They (54) have prepared a new nomogram for urine, accurately expressing total carbon dioxide as a linear function of pH and carbon dioxide tension (or H_2CO_3 concentration). Since it is theoretically exact, a similar nomogram constructed for serum (with appropriate values of α and pK') should be very useful. Indeed, such a relationship exists in the theoretically exact nomogram presented by Hastings & Shock (56). However, the values of the unknown factor are obtainable from the other known variables only indirectly, in two steps. For blood, their nomogram is limited to use with arterial or capillary samples. The earlier line chart of Van Slyke & Sendroy (57) for estimation of serum total carbon dioxide content from whole blood carbon dioxide content is applicable to blood of any degree of oxygen unsaturation. Van Slyke (58) has drawn a nomogram convenient for use in quickly estimating the dosage of sodium bicarbonate required to raise the alkaline reserve of the individual in conditions of alkali deficit. The necessary known factors are body weight and plasma carbon dioxide content.

Hastings & Steinhaus (59) have plotted pH, bicarbonate, and carbon dioxide tension of serum on triaxial co-ordinates. Displace-

ments of acid-base balance, both in direction and extent, are readily followed and characterized (60). An identically similar chart has been used by McClendon (61). Van Slyke (58), on the basis of additional data, has revised his logarithmic chart, indicating by areas, various conditions of acid-base balance.

OTHER ACID-BASE BALANCE METHODS AND APPARATUS

Shock & Hastings (62) have described a microtechnique for the determination of the percentage of cells, serum pH, and total carbon dioxide content of blood on a 0.1 cc. sample of finger blood. Sendroy, Seelig & Van Slyke (63) have adapted the direct measurement of carbon dioxide tension and pH of whole blood (64) to the analysis of urine acid-base balance. Barcroft (65) has devised a convenient type of equilibrating tonometer with self-contained pipette. Vessels for the storage of gases and liquids, suitable for various analytical procedures, have been described (66).

The problem of keeping the pH of freshly shed blood from changing before analysis was brought into sharp focus by the observation of Havard & Kerridge (67) of an apparent sudden fall in the pH of blood within a few minutes after being drawn. This phenomenon has been reinvestigated by them and others. That lactic acid formation takes place in freshly shed blood kept at 38° has been known for some time. That there is an accompanying fall in pH of about 0.03 to 0.05 during the first half-hour after bleeding has been amply confirmed by Laug (22, 68), Yoshimura (69), and Haugaard & Lundsteen (70). However, a "first acid change" in blood pH of the magnitude reported by Havard & Kerridge is too great to be accounted for by the amount of lactic acid formed during that time, and has rarely been observed by others. The explanation seems to be that such sudden changes of pH are a temperature artifact caused by initial differences in temperature between the electrode and the blood sample. When the temperature is carefully controlled the "first acid change" is not observed and only the more gradual drop in pH caused by glycolysis takes place (68, 71, 72, 63, 73). The effect on blood pH of anticoagulants and clotting has also been studied (68, 69, 74, 19, 75, 76) but could bear further investigation under carefully controlled conditions. It would be well to study the effects of clotting and anticoagulants on general acid-base balance and electrolyte equilibria, with variation in species, under varying conditions for the preservation of the sample, and at different pH values of the samples.

It is obvious from the above that almost all values in the literature of the pH of blood as drawn must be at least 0.03 pH more acid than in the circulatory system. For comparative clinical purposes this is of no serious consequence. At the present time, the best procedure for accurate determination of blood pH would seem to be to use sodium fluoride and potassium oxalate as anticoagulants, to draw the blood at 38°, and to determine the pH of the whole blood in the glass electrode (12) immediately. The next best procedure is to keep the blood as cool as possible until time for analysis, which should be done with the minimum of delay (2).

pH.
of blood

ACID-BASE BALANCE STUDIES OF BLOOD UNDER VARIOUS CONDITIONS

Most of the vast array of experimental and clinical data on acidosis and alkalosis which has appeared during the last few years covers ground already explored by earlier workers, whose observations have been reviewed by Austin & Cullen (77) and treated in detail by Peters & Van Slyke (1). Van Slyke (58) has reviewed the chemistry of acidosis and alkalosis, from the clinical standpoint, in what is probably the clearest presentation of the subject available at the present time. In this field, the more important recent contributions have added to a better understanding of acid-base balance changes, rather than to the accumulation of new facts. The consequent refinement of treatment of data with which many of the papers are concerned makes possible little more than a cursory survey of the literature on the subject.

Normal variation.—Shock & Hastings (78) have reinvestigated the change in acid-base balance of blood in normal individuals. They found a slight difference in average pH, bicarbonate, and carbon dioxide tension between the sexes. Fluctuations within a given individual might take place within the entire range of values found for normals; in some persons there was little fluctuation. They were unable to find any general characteristics in the diurnal variation. Their evidence indicates that significant variations of acid-base balance are a function of the individual. Looney & Jellinek (79) have studied the oxygen and carbon dioxide content of arterial and venous blood in normal individuals. Their values were a little lower for oxygen and higher for carbon dioxide than the averages of previous values in the literature. This they attribute to their technique of taking samples directly into a syringe containing sodium fluoride

and potassium oxalate. The samples were kept in the syringe until they were analyzed.

All of our acid-base balance studies on blood as drawn, normal and pathological, need reinvestigation. Most of the values for serum pH have been obtained by a colorimetric method, less frequently by indirect calculation from gasometric measurements. The possible deficiency of the colorimetric method has been discussed (p. 233). Gasometric results by interpolation (80) or by the direct carbon dioxide tension method (64) lack the desired accuracy. Furthermore, all previous work has been done without sufficient precaution against glycolysis and lactic acid formation. New data, obtained somewhat in the way suggested for pH determination (p. 239), would not only provide a more satisfactory normal standard, but might help to narrow the range of variation.⁶

Experimental and pathological variations of the acid-base balance of blood.—Van Slyke (58) and Shock & Hastings (60) have elaborated on the characterization and interpretation of respiratory and metabolic disturbances of the acid-base balance. The latter authors have studied the details of acid-base shifts, and the physiological significance of their direction and extent. From their results they conclude that individuals may be characterized in terms of the rate at which the acid-base balance of the blood is restored to normal after experimental displacement.

Alcoholism.—In alcoholism, chronic and experimental, there is a tendency to an acidosis of the metabolic type, characterized by an alkali deficit, probably the result of a production of organic acids (82, 83, 84). This is in accord with Palthe's (85) proof that acute alcoholism is a state of anoxia. In the extremes of the latter state, a corresponding acidosis results (86). Cellular hypersensitivity of the brain in rats is increased in experimental acidosis and decreased in alkalosis (87).

Cancer.—Investigators of cancer problems continue to seek a correlation between tumor growth and acid-base balance. Slight variations from normal during periods of tumor growth have been found on both the alkaline and the acid side by many workers too

⁶ In fact, data on this point have already been obtained by the technique mentioned on page 239 by Van Slyke, Belcher, Hiller & Alving (81). However, because of the uncertainties with regard to the glass-electrode pH measurements of whole blood at that time, the results were not considered satisfactory.

numerous to mention. Selvaggi (88) found blood pH to vary in either direction depending on the type of tissue involved in the cancerous condition. The question is apparently still not settled (89), probably because of the reason that whatever deviations from normal (in acid-base balance) may be found in cancer are secondary manifestations of other phenomena. In this connection, claims of a 90 per cent probability in the diagnosis of cancer when the blood pH is over 7.38, and of 95 per cent probability in diagnosis excluding cancer when the pH is below 7.36, seem somewhat amazing (90).

Diabetes.—In the acidosis of diabetes although the clinician now has available in addition to bicarbonate therapy that of combined administration of insulin, glucose, and salt solution, skillful handling of the patient requires blood analyses during the course of the rapid changes in acid-base balance. Kydd (91), and Peters, Kydd, Eisenman & Hald (92), have studied further the chloride loss associated with diabetic ketosis. The latter workers have theoretically considered the possibility of recombination with carbon dioxide of the bicarbonate base which has been displaced by ketone bodies. Albers (93) has called attention to the cessation of gastric hydrochloric acid secretion associated with the hypochloremia of diabetic acidosis, and the relatively depressed gastric acidity in diabetics treated with insulin. Korányi and Szent-Györgyi (94, 95) regard diabetes as primarily a disturbance not of fat metabolism, but of carbohydrate, and have recently advocated the administration of succinic acid as an antiketogenetic substance in diabetic acidosis. Krebs (96) claims, on purely theoretical grounds, that citric acid should be more effective. There is not yet enough evidence to determine the efficacy of this type of treatment. When *r*-sodium lactate, in place of bicarbonate, is used with insulin and salt, Hartman (97) omits glucose in the treatment of severe diabetic acidosis. Joslin *et al.* (167), in a study of 318 cases, finds his treatment inferior to theirs.

Exercise.—Certain aspects of acid-base metabolism in exercise have already been covered in this series of reviews in connection with the subject of muscular chemistry, and will be omitted here (98, 99, 100). Rice & Steinhaus (101) have called attention to possible errors when knowledge gained from experiments with animals is applied to the physiology of exercise in human beings. In man, exercise on the ergometer leads to acidosis with lowered pH and bicarbonate, and increased carbon dioxide tension. In dogs under similar conditions (treadmill running) the consequent increase in body temperature

results in an increased pH and reduction in bicarbonate and carbon dioxide tension. When excess heat was dissipated by swimming in water at 20°, an acid-base balance condition similar to that of man was reproduced. Several papers (102, 103, 104, 105, 106) have dealt with the effect of work during different states of acid-base balance. Dennig *et al.* (102, 103, 107) found that the ability of human beings to do vigorous exercise, and the capacity of the blood to neutralize lactic acid were impaired during a state of acidosis. In dogs, the acidosis (105, 106) of severe exercise was not regarded as a causal factor of fatigue because of the finding (contrary to that in man) that an initial imposed alkalosis often reduced the capacity for muscular exercise. The converse effect was noted in acidosis. Blood lactic acid formation was promoted in exercise during alkalosis. Malnutrition in dogs slowed up recovery of normal acid-base balance after fatiguing exercise (108).

Indicative of another functional adjustment of the body to a change from normal is the observation of Drastich, Adams & Hastings (109) that a reduction of as much as 50 per cent of active pulmonary tissue by pneumectomy caused no difficulty in respiration during exercise. Partial collapse of an equivalent amount of lung caused greater changes in acid-base balance than did partial pneumectomy.

In a study of a trained athlete, Hastings, Dill & Edwards (110) found a great excess of carbon dioxide in the tissues of the leg. The carbon dioxide was rapidly eliminated in the first six minutes following exercise. Lactic acid, greatly above normal, continued to increase during that time, then decreased. The physiology of heavy, and of continued, moderate work is receiving much attention now in Russia, undoubtedly on account of the Stakhanoff movement. Up to the present time little has been found that is new.

Diet.—The effect of diet tends to be minimized, as accumulating evidence continues to show that no appreciable alteration of the internal acid-base balance by diets of an acidifying or alkalinizing nature is possible. Only a few of the many papers which have more recently been published on this subject may be noted here. The large doses of ammonium chloride and sodium bicarbonate which, as is well known, are necessary to produce experimental acidosis and alkalosis would lead one to surmise what Bischoff, Sansum, Long & Dewar (111) have well shown, namely, that no significant change in blood pH or acid-base balance can be caused by the ingestion of large amounts of acid- or alkaline-ash foods. Cape & Sevringhaus (112) have shown

that the effects of equivalent doses of sodium bicarbonate and sodium citrate are similar, and transitory unless huge doses are used. They conclude that little may be expected of orange-juice therapy unless the daily administration of several liters is continued for a long time. No correlation between the nature of the diet, as regards potential acidity or alkalinity, and susceptibility to caries has been obtained (113, 114, 115). Bol (116) believes that a constant rise of alkalinity of the saliva, following the ingestion of oranges or apples, efficiently neutralizes acids which cause caries. However, Krasnow's analyses of saliva (117) for pH have failed to reveal any basis for the differentiation between caries-free and caries-susceptible individuals.

Pregnancy.—Acid-base balance in pregnancy is ordinarily slightly displaced in the direction of a decrease in plasma bicarbonate and carbon dioxide tension. Previous differences between laboratories (118, 119, 120, 121) as to the significance of slightly elevated pH values in normal and toxemia pregnancy have probably been largely exaggerated by errors in method (122, 123, 124). Nice, Mull, Muntwyler & Myers (123) more recently have found a lowered bicarbonate and total base change during normal pregnancy, associated with a mean antepartum pH of 7.41, electrometrically determined. [Shock and Hastings (78) found pH values in normal women to average 7.42, colorimetrically determined.] According to Kydd, Oard & Peters (118) and Zimmerman & Peters (125), when definite disturbances of acid-base balance are found in the toxemia of pregnancy (126, 127, 128) they are secondary and referable to pathological conditions (of vascular and renal origin) to which the state of pregnancy is a predisposing factor.

Renal impairment.—For an adequate account of acid-base balance changes in conditions of renal impairment the reader is referred to Peters & Van Slyke (1) and Peters (3). No direct evidence seems to have been found to support the theory that organic acids play any significant role in the production of nephritic acidosis. The study of Greene, Wakefield, Power & Keith (129) lends support to the contrary view that, in association with loss of base, there is also an accumulation and retention of phosphates and sulfates, due presumably to renal insufficiency (130). They found no accumulation of organic acids in the blood. Brull & Roersch (131), it is true, in acid-base balance studies of experimental uranium nitrate nephritis in dogs, have obtained results which give evidence of an increased concentration of organic acids in blood and urine. When normal kidneys were perfused

alternately with the blood of a normal and a nephritic dog, a greater excretion of organic acids in the urine was caused by the nephritic blood (132). Too little is known of other effects of uranium nitrate on the organism to translate the results of such experiments on dogs to studies of nephritis in humans. Alkali therapy in renal disease has been studied further by Way & Muntwyler (133) and by Huguenin, Sannie & Truhaut (134, 135, 136).

Rickets.—In rats, Shohl *et al.* (137) showed that the acidity or alkalinity of the diet was only a secondary factor in the production of rickets. Morris (138, 139), in a study of children with rickets complicated by tetany, could find no indication that infantile tetany and rickets are directly due to acid-base balance displacements in the body. Shohl (140) has recently demonstrated that rickets may or may not be produced on diets of different calcium: phosphorus ratio, depending on the absolute amounts fed. The change in all of the diets, with respect to rachitogenic power, was increased when mixtures of ammonium chloride and ammonium carbonate were added. Citric acid with sodium citrate had the opposite effect, abolishing the production of rickets by the previously rachitogenic diets. Since citrates and tartrates alone, of the several organic acids tested, prevented rickets, Shohl concludes that acid-base effects cannot be the sole factor determining their influence on the disease. Diminished alkali reserve has been observed in strontium "rickets" (141). Rickets complicated by chronic nephritis resulted in acidosis which Salvesen (142) was able to abolish by treatment with calcium lactate, with or without cod-liver oil.

Respiration.—The relationship of respiration to acid-base balance has been reviewed in detail by Peters & Van Slyke (1). By blood pH and alveolar carbon dioxide determinations on dogs, Winterstein (143) has found more evidence in support of the theory that pH, rather than carbonic acid concentration, is the controlling factor in the stimulation of the respiratory center. Shoji, Yoshimura, Saito & Fujimoto's (144) studies of men at rest failed to show control of lung ventilation by arterial or alveolar carbon dioxide tension. Gesell & Moyer (168) find that the rate and depth of breathing are importantly determined by the balance of rate and depth-promoting reflexes and by central chemical control. In asthma, alkalosis caused by hyperventilation (145) was observed. No extreme variations from normal were found in cases of pulmonary tuberculosis (146). Main (147) has studied alterations of alveolar carbon dioxide tension and blood

pH in man accompanying postural change. He found an increased pH and decreased alveolar carbon dioxide tension in the standing position as against the recumbent and ascribed the result to pulmonary overventilation. His results, he concluded, were not in accord with the idea that alveolar carbon dioxide tension is maintained within limits that may be regarded as physiologically constant. Contrary to the findings in the case of controls, major variations of the blood pressure during pulmonary hyperventilation in essential hypertension were not related to the shift of the acid-base balance (148).

The development of acute mountain sickness has again been studied recently in the Andes by Barron, Dill, Edwards & Hurtado (149). This affliction is generally ascribed to oxygen want or a condition of pure "anoxic anoxia" (1). The process of adaptation by hyperventilation to increase the oxygen in alveolar air results in a condition of alkalosis from carbon dioxide deficit, with a partly compensating fall in alkali reserve. That this acid-base shift is not the major factor in the process of adaptation was shown by the ineffectiveness of ammonium chloride in preventing mountain sickness. Barron *et al.* (149) stress the point that the tissue-oxygen transport system plays a greater role in determining the appearance of mountain sickness than does the vascular oxygen transport system. The diminished alkali reserve operates to increase the oxygen transport of the latter. On the other hand, the relative oxygen affinities and rate of combination of the oxygen with myoglobin and the hemoglobin, respectively, in the two systems, are such as to justify their conclusion concerning the relative importance of the two systems in high altitude adaptation.

Gastric disorders.—Since gastric secretion is derived from the body, gastric disorders may be reflected in the acid-base balance picture. However, it seems that striking alterations are found only in cases of profound disturbance, such as the alkalosis associated with persistent vomiting and loss of hydrochloric acid. Brunton (150) found no difference, outside of the experimental error, in pH of the arterial blood analyzed before and after gastric secretion. On the other hand, neither oral ammonium chloride acidosis in man (151) nor extreme sodium bicarbonate alkalosis in dogs (152) was found to have any effect on gastric acidity. In spite of previous contrary evidence, the gastro-intestinal tract is therefore regarded by these workers as a stable receptor in which deviations are produced only by profound changes (151, 153). Thiele (154) has found by analysis what he interprets as a direct relationship between gastric

acidity and blood calcium. Upon oral and intravenous administration of calcium he obtained a considerable increase in gastric hydrochloric acid. Debrue & Lacquet (152) also regard calcium as a participant in the regulation of gastric secretion. Schiffers (155) found, in the hypocalcemia caused by injections of disodium phosphate, a decrease in gastric acidity, whereas that of orthophosphoric acid had no effect. His conclusion that the calcium-ion concentration of the blood was the responsible factor governing the gastric acidity is in harmony with the studies mentioned above. Since calcium is low and relatively constant in concentration in gastric juice during fluctuations in acidity, and since the acidity depends entirely upon the concentration of fixed base excreted, more direct evidence on this point is needed. For related aspects of gastric and other alimentary secretions, the reader is referred to the treatment of Peters (3).

Ether anesthesia.—The observations of Van Slyke, Austin & Cullen (156) that both bicarbonate and pH of the blood are reduced in ether anesthesia have been confirmed (158, 159, 160, 161). Alkali administration counteracted the acidosis and deepened the anesthesia (160). A similar result was obtained in frogs in fluids of different pH (162). It is well known that morphine produces an acidosis of primary carbon dioxide excess, with lowered pH of the blood in man. Its action was found to be similar in dogs (163), but apparently the opposite in rabbits, in which a rise in alkali reserve (164) and increase in pH (160) was observed. Dallemagne (165) has reviewed the question of anesthesia as related to acid-base balance.

ACID-BASE BALANCE OF URINE

By the process of simple diffusion one would expect the pressure of dissolved gases to be equalized across the surface or membrane separating two liquid media. That this is true in the case of fluids ordinarily considered to be in osmotic equilibrium with blood has been known for some time in the case of lymph, spinal fluid, and transudates, the carbon dioxide tension of which is close to that of arterial blood (1). The results of Gamble (166), who found the carbon dioxide tension in urine to average 61 mm. within a comparatively narrow range regardless of the bicarbonate concentration, were in accord with the corresponding assumption concerning urine and blood. The carbon dioxide tension of arterial blood is very close to 40 mm., and averages a little less than 50 mm. for mixed venous

samples. Mainzer & Bruhn (55), however, found no such constancy in the results of a series of freshly voided urines, the carbon dioxide tension of which, calculated from data applied to the Henderson-Hasselbalch equation, showed a wide variation of from 13 to 242 mm.

In order to shed more light on the question, Sendroy, Seelig & Van Slyke (63) applied a method based on a familiar principle of direct carbon dioxide tension measurement, which eliminated the uncertainties of indirect determinations. In the main, their results, which yielded an average carbon dioxide tension of 67 mm., confirmed those of Mainzer & Bruhn. However, in not one of the sixty samples of normal and pathological human urine was there found a tension lower than 42 mm.—the arterial level. The upper limit was about 150 mm. except when the acid-base balance was altered by severe exercise or ingestion of sodium bicarbonate. Moreover, the higher carbon dioxide tensions were found only in concentrated samples stored in the bladder for a short period. Dilute urines, stored in the bladder for a long period, tended, either by the production of an average mixture from successive high and low excretions, or perhaps by some diffusion of carbon dioxide through the bladder wall, to approach the values found by Gamble.

With the aid of other necessary data, results for complete acid-base balance were described by Sendroy, Seelig & Van Slyke (63) and found to be in accord with the filtration-reabsorption theory of urinary secretion. On the basis of presumptive evidence afforded by comparative analyses of the pH and the concentration of other solutes in plasma and glomerular filtrate, as found by previous workers, carbonic acid and bicarbonate may be regarded as being filtered unchanged in concentration. Therefore, acidification of the urine in the tubule and the smallness of the amounts of bicarbonate found in the urine can be explained by the selective tubular reabsorption of bicarbonate. By contrast, the reabsorption of carbonic acid is merely by passive diffusion in the water reabsorbed by the blood. This would account for the fact that the minimum carbon dioxide level in urine seems to be that of the arterial blood.

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THE HORMONES*

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The authors have availed themselves, perhaps too freely, of the privilege extended to them by the editors to select from the immense bulk of current endocrinological literature certain subjects which are close to their own fields of interest. They wish to express their regret to the many meritorious investigators in other fields, whose work has not been included. They also apologize for the unavoidable oversights, and possible misinterpretation of results, in the subjects treated.

ADRENAL CORTEX

The continued interest in the manifold problems presented by the adrenal cortex is manifest in the impressive volume of work that has appeared during the review period. Outstanding success has been accomplished in the chemical field. Although progress along physiological lines was less spectacular, its presentation here will serve to supplement the excellent résumé of Marrian & Butler in the hormone chapter of last year's review.¹

Physiologically active compounds.—The past year has brought clarification into the controversy concerning the identity of the physiologically active principle in cortical extracts. The existence in the gland of several chemically related compounds, each of them exhibiting "cortin" action in the accepted testing methods, seems now to be well established. Compound H of Reichstein, already isolated in 1936 (1) in slightly impure state, and now termed corticosterone, was found to be active in the Everse-de Fremery test (0.5–1 mg. per rat) and in the adrenalectomized dog (maintenance dose 1.25–2.5 mg. per animal per day) (2). Shortly afterwards Kendall *et al.* (3) recognized the identity of their Compound B, after proper purification and revision of its formula, with corticosterone. The

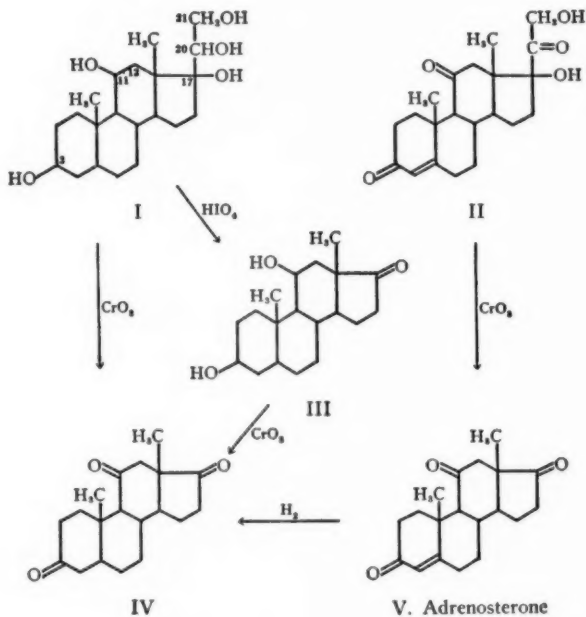
* Received January 25, 1938.

¹ *Ann. Rev. Biochem.*, **6**, 303 (1937)

daily maintenance dose in the dog test is given as 1.5 mg. per animal (3), the minimum effective dose in the Ingle rat test as 0.1 mg. (4). Two other compounds of the Kendall series, A and E, also exhibit physiological activity (5). Compound A, 11-dehydrocorticosterone, is somewhat less active physiologically than corticosterone (4, 5). Reichstein (6), who obtained it from corticosterone by chemical transformation, has as yet not reported on its physiological efficacy. Compound E of Kendall was reported as active in the Ingle test in a dose of 2 mg. daily (7). Negative results, probably due to insufficient dosage, had been obtained with this compound by Reichstein (1) in the Everse-de Fremery test (0.8 mg. daily), and by Wintersteiner & Pfiffner (8) in the dog assay (0.1 mg. per kg. per day). A fourth compound, M of Reichstein, also appears to possess activity according to this author (9). It is unlikely that the compounds enumerated account for the full physiological activity of the extracts, since all three investigating groups have obtained non-crystalline fractions considerably more potent than the compounds thus far isolated (1, 5, 8). According to Kendall *et al.* (5) fractions of this type, which are at least ten times as potent as corticosterone, contain mainly compounds of the O_5 -series.

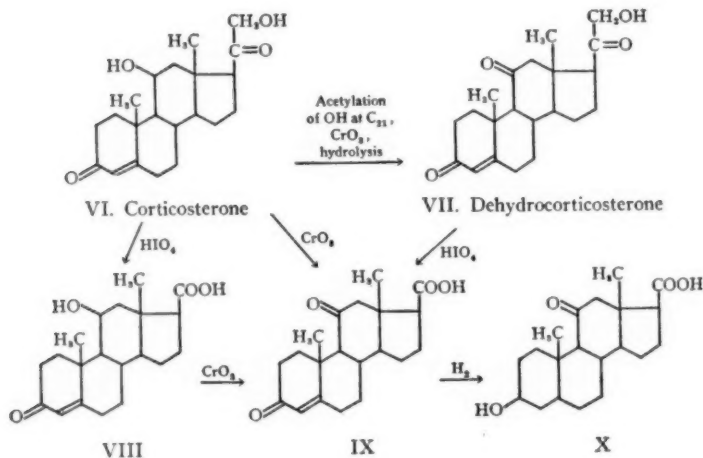
Chemical structure.—Structural analysis of adrenal compounds has now progressed far enough to merit discussion. Only the compounds of the O_5 -series ($C_{21}H_{30}O_5$) and O_4 -series ($C_{21}H_{30}O_4$) are well investigated. For the former series the proof of structure can now be considered complete, thanks mainly to the brilliant work of Reichstein (1, 9, 10, 11, 12). The O_5 -compounds are characterized by the ease, due to the presence of a tertiary hydroxyl, with which oxidative fission takes place at the point where the side chain is attached. On treatment with chromic acid they yield ketones, $C_{19}H_{26}O_3$ or $C_{19}H_{24}O_3$, depending on whether the original compound is saturated or unsaturated (Formulae I to IV, II to V). Reichstein has now shown conclusively that these substances, in which only two keto groups are demonstrable with the usual reagents, are actually triketones (12). They possess androgenic activity about equal to that of androstenedione. The unsaturated triketone V (adrenosterone, also isolated from the gland) can be hydrogenated to IV, and the latter eventually converted to androstane-17-one (proving the position of one of the oxygens), and to androstane, which establishes the structure of the ring skeleton. Less drastic oxidation of I, with lead tetraacetate or periodic acid, leads to a monoketone (III). The

amount of reagents used up in the latter reactions points to the presence in the original compound (I) of a glycerol grouping, one of the hydroxyls of which must be tertiary and remains as carbonyl after the elimination of the two-membered side chain. The monoketone III is digitonin-precipitable, which fixes the position of one of the



hydroxyls in this compound, and therefore of the second reactive keto group in IV, at carbon atom 3. The single (reactive) keto group in III, and consequently the side chain eliminated in its formation, must therefore have position 17 already ascertained for one of the oxygens in IV. Thus the presence in the original compounds (I, II) of an allopregnane- or pregnene skeleton substituted at positions 3, 17, 20, 21 may be considered as established (as to function and position of the fifth, "inert" oxygen, see below).

Such direct and practically complete proof of structure is still lacking for the compounds of the O_4 -series although, thanks to the efforts of Kendall and coworkers (3, 4) and of Reichstein (6), most structural details have been elucidated, and there is little doubt that the proposed formulae are essentially correct. These compounds do not give rise to androgenic ketones on oxidation, but yield acids with the elimination of only one carbon atom. Thus corticosterone (VI) gives an acid $C_{20}H_{28}O_4$ (VIII) with periodic acid, dehydrocorticosterone (VII) the corresponding acid $C_{20}H_{26}O_4$ (IX). Chromic acid converts corticosterone directly to the latter (VI to IX). This behavior reveals the absence in the O_4 -compounds of the C_{17} -hydroxyl, which makes the oxidation of the O_5 -compounds to androstane derivatives possible. The position of the side chain at C_{17} is therefore only assumed by analogy. The observation (4) that acid



IX can be reduced catalytically (directly or in two steps) to the saturated digitonin-precipitable acid X has been interpreted as evidence for the steroid nature, and, by further inference, for the 3-position of the keto group. Acid X is also formed by side chain oxidation of Compound H (isolated) of Kendall, which is also precipitable by digitonin. Kendall and coworkers present evidence that acid X corre-

sponds sterically to β -cholestanol; this is entirely analogous to the findings of Reichstein concerning the saturated O_5 -compounds.

The chemically inert, hitherto uncharacterized oxygen atom present in both series has been finally revealed as a secondary hydroxyl in some compounds, as carbonyl in others. Kendall *et al.* (3) first succeeded in characterizing, by means of the derived acids, this oxygen atom as hydroxyl in corticosterone (VIII to IX). Reichstein employs a more direct method (VI to VII), which is also applicable to the analogous α -ketols of the O_5 -series; other O_5 -compounds require degradation to C_{19} -ketones for characterization of the inert group. The position of this group is still somewhat uncertain. Position 12, and later 11, both of which are known to be sterically hindered, were proposed by Kendall in his formulae for corticosterone (3, 4, 13). Reichstein favors position 11, and has adduced satisfactory, though indirect, evidence for the correctness of this view, involving transformations in the C_{19} series of ketones (12).

A most convincing argument for the essential correctness of the structure proposed for corticosterone has evolved from synthetic attempts. Steiger & Reichstein (14) succeeded in preparing a compound possessing cortin activity equal to that of corticosterone from Δ^5 -3-acetoxy-aetiocholenic acid, obtained by degradation of stigmasterol. The new compound, desoxycorticosterone, is a progesterone substituted at C_{21} with an hydroxyl. Its structure differs from that proposed for corticosterone only by the absence of the "inert" 11-hydroxyl. This important result shows that the latter group is not essential for the physiological action. The 21-hydroxyl, on the other hand, is necessary, since progesterone possesses no cortin activity. The essential nature of the double bond is attested by the findings of Kendall and coworkers (4) that its hydrogenation in corticosterone and dehydrocorticosterone largely destroys the activity.

Table I (p. 258) attempts a classification, on the basis of structure, of the steroid compounds isolated from cortical extracts.

The search for pathological excretion products in the urine of patients exhibiting various manifestations of the adrenogenital syndrome has yielded results of considerable interest. The compound $C_{21}H_{30}O_3$, which was isolated by Butler & Marrian (15) from the urine of two female subjects with symptoms of virilism, was shown to be pregnane-3,17,20-triol. Its derivation from the adrenal can be hardly doubted since it disappeared from the urine after removal of

the enlarged gland. It was not stated whether this compound shows male-hormone activity, but its structure would seem to preclude androgenic properties. The high male-hormone content of the urine

TABLE I*

STEROID COMPOUNDS ISOLATED FROM CORTICAL EXTRACTS

Nomenclature of R. K. P.W.			Formula	Proposed structure	Remarks
A	D	A	$C_{21}H_{34}O_5$	Allopregnane-3,11,17,20,21-pentol	(I)
C	C	D	$C_{21}H_{32-34}O_5$	Allopregnane-3,11,17,21-tetrol-20-one	Differ by function (hydroxyl or carbonyl) of C_{11} oxygen
D	G	..	$C_{21}H_{32-34}O_5$	or Allopregnane-3,17,21-triol-11,20-dione	
E	$C_{21}H_{32}O_5$	Δ^4 -Pregnene-11,17,20,21-tetrol-3-one	
M†	F‡	..	$C_{21}H_{30}O_5$	Δ^4 -Pregnene-11,17,21-triol-3,20-dione	
Fa	E†	F	$C_{21}H_{28}O_5$	Δ^4 -Pregnene-17,21-diol-3,11,20-trione	(II)
..	H	..	$C_{21}H_{32}O_4$	Allopregnane-3,21-diol-11,20-dione	Corticosterone (VI) Dehydrocorticosterone (VII)
H†	B†	..	$C_{21}H_{30}O_4$	Δ^4 -Pregnene-11,21-diol-3,20-dione	
..	A†	..	$C_{21}H_{28}O_4$	Δ^4 -Pregnene-21-ol-3,11,20-trione	
J	$C_{21}H_{30}O_3$		Probably a tri-atomic alcohol
L	..	G‡	$C_{21}H_{32-34}O_3$		Monoketone
G	$C_{19}H_{28}O_3$	Δ^4 -Androstene-3,11,17-trione	Adrenosterone (V) (androgenic); also by degradation of unsaturated members of O_4 -series

* Compiled from a comprehensive review by Reichstein [*Ergeb. Vitamin Hormonforsch.*, 1, 334 (1938)]. The authors wish to express their appreciation to Professor Reichstein for permitting them the use of the manuscript.

† Cortin action.

‡ Identity not certain.

Compound K of Reichstein: found later to be identical with H (corticosterone).

Compound B of Pfiffner and Wintersteiner: difficultly separable mixture of O_5 -monoketones
Compound C of Pfiffner and Wintersteiner: not identical, as was previously assumed, with Reichstein's E, but hydrate of A (unpublished).

in a case of virilism investigated by Callow (16) was accounted for by the isolation of large amounts of isodehydroandrosterone. Burrows, Cook *et al.* (17) obtained a weakly androgenic substance, identified as $\Delta^{3,5}$ -androstadiene-17-one, and small amounts of two com-

pounds $C_{19}H_{28}O_3$ and $C_{21}H_{32}O_3$ (isolated as derivatives) from the urine of a male patient with an adrenal tumor, who exhibited symptoms of feminism. Further pursuit of such studies will perhaps permit correlation of the chemical findings with the variations in the clinical picture and glandular pathology encountered in these disturbances.

Bioassay.—Bülbring (18) recommends the use of the adrenalectomized drake, on account of its short survival period, for rapid assay of the cortical hormone. Gaarenstroom *et al.* (19) devised a new form of the muscular fatigue test, in which adrenalectomized rats are forced to swim till exhaustion supervenes. Schultzer (20) describes a rat-assay method, based on measuring the percentage of survival under well-standardized conditions. Cartland & Kuizenga (21) compared their rat unit, based on percentage of survival and maintenance of growth, with the dog unit of Pfiffner, Swingle & Vars. Their results adduce precise proof for the hitherto only surmised fact that the cortin requirement of the rat, on the unit weight basis, is very much greater than that of the dog.

Physiology.—While till recently the action of the cortical hormone could be demonstrated only by reversing the abnormal changes resulting from impaired cortical function, it has now been shown repeatedly that large doses of cortin will influence the electrolyte metabolism of the normal human subject (22, 23) and of the dog (24, 25). The changes in electrolyte excretion occur in the same direction as those observed after hormone therapy in adrenal insufficiency (i.e., diminution of urinary sodium and, less constantly, of chloride, and increased elimination of potassium) but are less marked quantitatively. Withdrawal of the hormone after sustained treatment results in a "rebound" effect, e.g., more sodium and less potassium is now excreted than during the control period. The marked effect of cortin on electrolyte excretion is, however, not reflected in any significant changes in the concentration of the plasma electrolytes. That the electrolyte distribution in the blood may be influenced, under pathological conditions, by an excess of hormone, is suggested by a report of McQuarrie *et al.* (26), in which a case of Cushing's syndrome associated with a persistent tendency towards alkalosis, high blood sodium, low blood potassium and chloride, and hyperglycemia is described. These blood changes, in most respects opposite to those found in Addison's disease, were interpreted as indicating over-function of the adrenal cortex. Anderson & Haymaker (27) noted

that when serum of patients suffering from this syndrome was injected into adrenalectomized rats, the average survival period of these animals was about double that of the controls receiving normal serum.

The primary disturbance in this syndrome may well be in the pituitary. While direct evidence that the adrenotropic hormone of this gland increases the output of cortin is lacking, it appears certain from the experiments of Ingle & Kendall (28) that a high level of cortin in the body fluids suppresses the secretion of adrenotropic hormone, as evidenced by the cortical atrophy which results from the injection of large amounts of cortin into normal rats.

The essential function of the cortex, and the relative importance of the various symptoms in the deficiency syndrome, are still matters of vigorous dispute. Zwemer & Truszkowski (29) reaffirm their position in regard to the corticoadrenal regulation of potassium metabolism by observations on the potassium tolerance of normal and adrenalectomized animals. The determination of blood-potassium tolerance curves is recommended as a means of diagnosing early Addison's disease (30). Leaving open the question whether the regulation of blood potassium is effected primarily by the kidneys or by internal shifts between tissue cells and body fluids, the authors point out that a change in the permeability of cell membranes toward potassium must be involved in either case. Essentially the same idea is advanced by Nilson (31), and by Ingle, Nilson & Kendall (32). The latter authors investigated the effect of cortin on the blood electrolytes and on the working capacity of adrenalectomized-nephrectomized rats. Of all the changes observed, the high concentration of serum potassium, and its decrease after cortin treatment, were considered as most characteristic. The working capacity, impaired in the untreated animal, is maintained by cortin. It is suggested that cortin, independently from its action on the kidney, "may be concerned with the permeability of the cells in regard to potassium, and that the effect of cortin on the capacity for work may be associated with its favorable effect on the distribution of potassium." However MacKay *et al.* (33), observing that the survival period of nephrectomized rats is cut to half by simultaneous adrenalectomy, discounts potassium retention as causing the earlier failure of the doubly operated animals.

The effect of water intoxication, a condition associated with low levels of serum sodium chloride and with fluid shift to the tissues, on

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adrenalectomized dogs was studied by Swingle *et al.* (34). The prompt recovery from the symptoms of water intoxication effected by cortin was found to be accompanied by a sharp rise, though not always to normal levels, of blood sodium and chloride, and by rehydration of the blood. The ions, in the absence of external sources, were thought to be derived from intracellular stores, and it is further assumed that their release osmotically affected the shift of fluid from intracellular to extracellular compartments and to the blood stream. The same mechanism, according to the authors, is operative during the recovery, after cortin administration, of adrenalectomized dogs which had been maintained on a salt-free diet and were brought into insufficiency by gradual withdrawal of the hormone (35). The pertinent observations in the latter investigations are: absence of significant changes in blood electrolyte levels while insufficiency develops; a slight fall of blood sodium chloride, probably caused by rehydration of the blood, following relief of the symptoms by injection of extract; and a subsequent rise during the recovery period in spite of further blood dilution, absence of food intake, and increased renal elimination.

That plasma volume can be restored by cortin in conditions other than adrenal insufficiency was shown by McAllister & Thorn (36) in experiments on etherized dogs, which exhibit marked haemoconcentration.

Further data on electrolyte and carbohydrate levels in blood and muscles of the adrenalectomized opossum are furnished by Britton & Silvette (37). The sodium chloride levels in such animals may deviate from the normal in either direction, depending on the alimentary conditions and state of hydration. These changes are interpreted as reflecting merely alterations in the renal and extrarenal excretion of salt, while it is emphasized that the carbohydrate stores in blood and tissues are invariably decreased. The same authors (38) find that in the cat reductions in serum sodium chloride, equal or greater than those observed after adrenalectomy, may result from pancreatectomy, nephrectomy and various combined operations. This is contrasted with the loss of total carbohydrate, which assumed more serious proportions after adrenalectomy than in any of the other conditions studied.

Laszt & Verzár (39) attempt to show that the disturbances in electrolyte metabolism following adrenalectomy are secondary to the impairment of carbohydrate utilization, which in turn is referred to

the general failure of phosphorylation processes. They showed that the oral ingestion of glucose, in amounts well tolerated by the normal animal, causes in rats with well developed insufficiency a fatal diarrhea accompanied by severe losses of sodium salts into the gut. After subcutaneous injection of glucose, adrenalectomized rats likewise succumb quickly. Injection of hypertonic saline and urea solutions did not have this effect. From these and other observations it is argued that in the body tissues of the adrenalectomized animal a similar inability to "take up" glucose may exist as in the intestinal wall, causing, osmotically, the loss of sodium salts and water into the blood stream, and that this mechanism may also be concerned in the renal loss of sodium known to occur in other species after adrenalectomy. It is not clear to the reviewers how the extremely low blood-sodium values recorded by the authors, and the absence of sodium loss in their balance experiments (both in insufficiency uncomplicated by glucose administration) can be reconciled with this hypothesis. The fact that administration of cortin prevents the collapse of the adrenalectomized, glucose-fed rat has been utilized for the bioassay of cortical hormone (40). Another publication from Verzá's laboratory (41) deals with the distribution of free and bound lactoflavin in the livers of rats with and without adrenals. In the normal animal by far the greater part (94 per cent) of lactoflavin is present in non-diffusible form, that is, according to current views, as lactoflavinphosphoric acid combined with the protein carrier of the yellow enzyme. Four days after adrenalectomy the ratio of bound to free lactoflavin is only 2:1 or lower, and the total lactoflavin is also much decreased, indicating that the animal has at least partly lost its ability to phosphorylate and thus to fix lactoflavin. However, the findings that administration of cortin failed to bring about a more normal distribution remains unexplained. The effect of varying doses of cortin and of lactoflavin, administered to adrenalectomized rats kept on a diet free from the latter, on growth and survival was investigated by Verzá & Laszt (42). The bearing of the results on the bioassay of cortical extracts is discussed.

Deuel *et al.* (43) found that the rate of glucose absorption and of glycogen deposition in the liver is not significantly lower in the adrenalectomized rat, well maintained by salt therapy, than in the normal rat. This seems to contradict the numerous observations of Verzá (39, and previous papers) indicating that the selective absorption of glucose from the intestines is impaired in the absence of the

adrenal, but it should be remembered that the Swiss workers used animals in a well-advanced state of insufficiency. However, it would appear from Deuel's results that the deficiency in glucose absorption evident in the latter animals is not referable to lack of life-maintenance hormone as such, but rather to the consequent disturbance of salt metabolism.

Previous observations demonstrating that adrenalectomy abolishes the ketosis and fat deposition in the liver induced by fasting, fat feeding, administration of ketogenic pituitary extracts and other measures have been confirmed and extended (44-47). Salt therapy does not modify the failure of ketogenic extracts to produce these effects (44). The presence of surviving cortical tissue permitted the ketogenic response to pituitary hormone, but cortin did not replace this function (47). While the latter observation is in line with previous experience (G. Evans; Long & Lukens), MacKay & Barnes (48) have succeeded in increasing the fasting ketonuria of normal female rats by administration of a cortical extract very potent in life-maintenance activity. Long, Lukens & Dohan (49) showed that in the cat, as in the dog, the glucosuria and acetonuria following pancreatectomy is markedly reduced after removal of the adrenals. The idea that neoglycogenesis is interfered with in the absence of the adrenal, receives support from the finding of Samuels *et al.* (50) that adrenalectomized rats failed to store glycogen in the liver after ingestion of alanine. The experiments of Zucker & Berg (51) bring out the fact that restitution of the normal blood-sugar level after insulin injection is markedly delayed in adrenalectomized cortin-maintained dogs as compared with normal or demedullated animals, proving that the cortex participates in the normal defense mechanism counteracting hypoglycemia. Here again amounts of cortin adequate for life maintenance apparently failed to bring about complete replacement of cortical function. Samuels *et al.* (52) found that the sugar-tolerance curve of adrenalectomized rats was somewhat elevated above the normal, and that this difference was abolished after cortin or salt therapy. The action of cortin was therefore ascribed to its beneficial influence on electrolyte metabolism rather than to a specific effect on carbohydrate stores.

The question as to the identity of endocrine factors in the gonadal-hypophyseal sphere, which, under certain conditions, seem to be capable of replacing the life-maintenance hormone, still remains unanswered. Swingle *et al.* (53) supplement their observations already

reported last year (54) by the demonstration that progesterone does not prolong the life span of adrenalectomized cats and dogs. Barring the possibility of insufficient dosage, it is therefore improbable that the protection afforded by certain gonadotropic preparations, and by pituitary implants [Emery & Schwabe, cf. *Ann. Rev. Biochem.*, **6**, 310 (1937)] is mediated through this hormone. The fact that castration did not abolish the beneficial effect of a certain pituitary preparation on the survival of adrenalectomized male cats rules out the participation of a testicular principle. Cavanaugh & Gaunt (55) could extend, but not indefinitely prolong, the life period of adrenalectomized female rats by moderate doses of homologous pituitary implants. Negative results were obtained with beef pituitary implants, pregnancy-urine preparations, and progesterone, while oestrogens were found definitely toxic.

It seems appropriate to mention in this connection the remarkable discovery of Thorn & Harrop (56) that various gonadal hormones (oestradiol, progesterone, testosterone) as well as pregnandiol, when injected into normal dogs, cause a marked diminution of renal sodium excretion. Before similar experiments have been carried out on animals without adrenals, hypophyses, or gonads, it is idle to speculate on the possible mechanism of this effect. The authors suggest that the sodium sparing effect of the ovarian compounds may explain the survival of the adrenalectomized bitch during oestrus and pregnancy. Swingle's negative results with progesterone do not support this idea, but the doses used in his maintenance experiments were probably too small to influence electrolyte excretion.

Davidson (57) showed that the enlargement of the seminal vesicles and prostate produced by the hypophyseal corticotropic hormone in the castrated rat also obtains in the castrated hypophysectomized animal, but the effect is not observed after removal of the adrenals. This clearly indicates that the adrenal cortex can be stimulated to secrete an androgenic principle. The results of Moon (58), who induced precocious sexual maturity in young female rats with the same adrenotropic preparation, are less conclusive, but invite an analogous interpretation. Corey, in experiments with hypophysectomized rats (59), and Fitzhugh (60), working with ovariectomized rats, could not demonstrate oestrogenic activity in adrenal extracts prepared by the Pfiffner-Swingle method. But it is surprising that extracts of this type should contain gonad-stimulating principles, as would appear from the findings of Fitzhugh (60), who could, by the use of such

a preparation, not only repair the degenerative changes taking place in the gonads of rats after adrenalectomy, but also produce ovarian and uterine hypertrophy in young female rats. The mode of preparation of these extracts would certainly preclude identity with the gonadotropic fractions obtained from adrenals by other workers, and more recently by Hoffmann (61). The adrenal gonadotropic principle of this author is insoluble in acetone, alcohol, and ether; it can be freed from proteins by ultrafiltration of its aqueous solution. This property distinguishes it from hypophyseal gonadotropic hormone, which it resembles, however, in its action on the rat ovary (weight increase, follicle stimulation, luteinization of the theca, augmentation by pregnancy urine).

Selye (62, 63) attempts a new interpretation of adrenal function, which is deduced from observations on the reaction of intact and adrenalectomized animals towards "alarming stimuli," such as exposure to low temperature, excessive muscular work, drugs, etc. Of novel interest are his results pertaining to adaptability toward alarm stimuli. Normal animals can gradually adapt themselves to such stimuli so that the latter cease to elicit the symptoms of the "alarm reaction," which are synonymous with those of adrenal insufficiency. Adaptation can also be acquired by adrenalectomized animals, which then become resistant to stimuli that otherwise would precipitate critical symptoms and collapse. The adrenals are conceived to play an important rôle in the first stage of adaptation, while later resistance is acquired by the peripheral tissues. It is furthermore assumed that the symptoms of the alarm reaction are due to liberation of a toxic metabolite, and that the adrenal detoxifies this substance. That adrenalectomized dogs can acquire tolerance toward ingested potassium salts has been noted by Kendall & Ingle (64). They deny, however, that the ultimate cause of the damage, i.e., the agent toward which resistance is acquired, is an endogenous toxin such as histamine. Furthermore, it has been demonstrated by Ingle (65), and by Wyman & Suden (66), that the increased susceptibility of adrenalectomized rats to histamine shock is a consequence of cortical as well as medullary deficiency. Experiments which would seem to lend support to the toxin theory are described by Riml (67) who showed that injection of serum or serum dialysates from adrenalectomized rabbits into normal guinea pigs causes fatal symptoms and pathological changes in the adrenals. The toxic factor is not present in normal rabbit serum.

TESTIS

In Koch's (68) thorough review of the chemistry and physiology of the male sex hormones a large part of the literature of 1936 is considered.

Hirano (69) has isolated several compounds from hog testes, of which only one, $C_{21}H_{32}O_3$, is a steroid. This substance, which is thought to be an allopregnane with an hydroxyl on C_3 and two carbonyl groups in the side-chain, is possibly related to the $C_{21}-O_3$ compounds present in adrenal extracts. The physiologically inactive diol isolated by Butenandt several years ago from human male urine was identified by Ruzicka *et al.* (70), and by Butenandt *et al.* (71) as *epiaetiocholane-3,17-diol*, which can be obtained by reduction of Δ^5 -testosterone benzoate, or by two-step hydrogenation of Δ^4 -testosterone. Among new androgenic products prepared in the laboratory the β , γ unsaturated ketones— Δ^5 -testosterone (only obtained in form of esters) (72), and Δ^5 -androstenedione (73)—are of certain interest, because they can be reduced to the difficultly accessible unsaturated *epi* compounds, *epiandrostenediol* (70) and *epidehydroandrosterone* (74), which are stereochemically related to androsterone.

A novel method to effect reduction of androgenic ketones is the use of fermenting yeast [Mamoli & Vercellone (75, 76, 77)]. Thus Δ^4 -androstenedione is reduced by yeast to testosterone; only the keto group at C_{17} is attacked, a result which would be difficult to accomplish by chemical methods. These studies have yielded valuable information about the influence of constitutional factors on the course of the enzymatic reduction of androgenic ketones by yeast cells, but it is a matter of mere conjecture whether these reactions actually reflect metabolic pathways of the androgenic hormones in the animal organism.

The influence of esterification on the androgenic activity of testosterone and other compounds has been extensively investigated (78 to 84). Following Miescher (79), the lower fatty acid esters of testosterone up to the propionate are most efficient in regard to comb-growth activity; with increasing chain length of the fatty acid radical the action becomes less intense, but prolonged. Tested in the castrated rat (seminal vesicles and prostate) the lower esters are many times more efficient than testosterone. A maximum of intensity and duration of action is reached with the normal- and iso-butyrate and the *n*-valerate; the efficiency then decreases with increasing chain length;

the palmitate and stearate are ineffective. Differential effects on the seminal vesicles and the prostate are apparent. Testosterone propionate may restore, under certain conditions, the accessory glands of the castrated infantile rat to a more normal state than testosterone, inasmuch as the weight of the seminal vesicle exceeds that of the prostate, as is the case in the normal adult rat. The enol diesters of testosterone, particularly the diacetate, have been tested by Ruzicka & Fischer (85), Deanesly & Parkes (86) and Miescher *et al.* (87). According to the latter authors the effect of the diacetate is less intense but more protracted, in the capon as well as in the ten-day rat test, than that of the monoacetate. Deanesly & Parkes (88) studied the influence of the mode of administration on the intensity and duration of action. They found that the effect of testosterone, implanted subcutaneously in solid form, exceeded that of the propionate injected in oil solution. It is suggested that the prolonged action of the esters is due to diminished solubility rather than to delayed hydrolysis.

Strong augmentation of action can also be brought about by activators added to the solution of the androgen to be tested. Miescher, Wettstein & Tschopp (89), prompted by Laqueur's observations on the presence of activating factors of acidic nature in testis and urine extracts which augmented the action of testosterone on the seminal vesicle of the castrated rat, discovered that the higher fatty acids are very efficient in this respect. A large number of saturated and unsaturated acids, most of them aliphatic, have been tested for their augmenting action (90). The former were generally found to be more effective. Maximum efficiency is attained with the acids possessing sixteen carbon atoms. The higher monovalent alcohols, especially stearic alcohol, also exhibit augmenting activity. The authors assume that the activating effect is brought about by diminishing the rate of absorption of the injected androgen from the tissue. That the mode of action is a purely local one is attested by the finding (89, 91) that augmentation does not occur when androgen and fatty acid are injected at different sites of the body. However, Polak *et al.* (92) hold that this explanation is not applicable to the natural activators (X-substances), which exert their effect in quantities not likely to influence the rate of absorption of the androgen.

Frank & Klempner (93) modified the comb-growth method of assay by measuring the weight increase of the combs of baby chicks. Voss (94) compared the relative efficiency of local application and of

intramuscular injection of androgens on comb growth. Zimmermann (95) studied the factors influencing the precision of his colorimetric method for the determination of androgens. The method has been modified and employed for urine studies by Wu & Chou (96), Callow *et al.* (97) and Oesting (98). The lack of correlation between the colorimetric values and bioassay results, apparent in the data of Oesting, would not seem to recommend the method in this particular form for anything but a very rough estimation of the male hormone content of urine.

Kochakian (99) gives data on the excretion of androgens in normal human males. Dingemans *et al.* (100) determined the male hormone content of the urine of children and adults of both sexes and include data on male hormone excretion during the menstrual cycle and pregnancy. The fact that excretion of male hormone persists, although on a much lower level, after cessation of sexual function, and after ovariectomy, is of particular interest. Callow (97), on the basis of similar observations, suggests that the adrenal may be the principal source of excreted male hormone. Thanks to the efforts of Koch and his group detailed information is now available on the daily excretion of androgens and oestrogens of normal individuals of both sexes (101), and of patients with various endocrine disturbances in the sexual sphere (102). There is no evidence of a cycle in the excretion of androgens or oestrogens in normal men, nor is there any regularity of fluctuations in the androgen content of female urine. Male castrates excrete only very small amounts of gonadal hormones. In virilism, the level of urinary oestrogen is generally low, while the androgen content is normal or high. Very high levels of androgen excretion were observed in cases of adrenal virilism. The conditions of acid hydrolysis necessary for obtaining maximal yields of both activities have been defined by Peterson, Gallagher & Koch (103). Dorfman *et al.* (104) report data on male and female hormone excretion in children. The variability of hormone excretion encountered within the same age group seems to be correlated with the degree of physical maturity.

Hooker (105) performed male hormone assays on testes of bulls ranging from one month to fifteen years in age. Since attainment of sexual maturity, as ascertained by the presence of motile spermatozoa, is not accompanied by an abrupt rise of the hormone content of the gland, it is suggested that the end organs acquire increased responsiveness to the hormone stimulus at puberty.

Hill's (106) highly interesting transplantation experiments reveal new facts bearing on the potential faculty of the ovary to secrete male hormone. He made the remarkable observation that mice ovaries, grafted into the ear of the castrated male, effect restitution of the seminal vesicles and prostate, while abdominal transplants failed to do so. It appeared a reasonable assumption that temperature, which in the ear, as in the scrotum, is several degrees lower than abdominal temperature, was the agent controlling the secretion of the male hormone from the grafted ovary. And indeed the male hormone effect could be enhanced by exposing the animals carrying grafts in their ears to low temperatures, while it was suppressed by environmental temperatures close to that of the body.

The maintenance of the seminiferous epithelium and of spermatogenesis in the hypophysectomized rat by the administration of male hormone urinary extracts, first described by Walsh, Cuyler & McCullagh, has been confirmed by Nelson & Gallagher (107). Their results are at variance with those of McEuen, Selye & Collip (108), who used 200 μ g. of testosterone daily, but failed to observe any maintenance of the tubules. That this was due to insufficient dosage is evident from the work of Cutuly, McCullagh & Cutuly (109), who could prevent the testicular atrophy following hypophysectomy with larger doses of androsterone or testosterone. They suggest that the action of these hormones on the seminiferous tubules is indirect, through maintenance of the scrotum. Nelson & Merckel (110) do not concur with this opinion. These authors tested seven crystalline androgens for their capacity to maintain spermatogenesis in the hypophysectomized rat, and found that their efficiency in this respect bears little relation to their effect on the scrotum. Thus, testosterone was found to be only moderately effective in maintaining testis' weight and spermatogenesis, in spite of its marked action on the scrotum.

The effect of androgenic compounds on the adult (83, 111, 112, 113), immature (114), and spayed (82, 107, 112) female rat has been extensively investigated. It is clear from these reports that androsterone, androstenedione, and testosterone, when given in sufficient amounts, suppress the oestrus cycle of the normal cyclic rat. Enlargement of the corpora lutea was noted by Nelson & Merckel (112) after the administration of testosterone and androstenedione. Wolfe & Hamilton (115) obtained the same result with testosterone, but only when treatment was started during oestrus and metoestrus. None of the androgens tested produced oestrus signs in the spayed

rat, but isodehydroandrosterone, and to a lesser extent *cis(epi)*androstenediol, markedly prolonged the oestrus period in the normal cyclic rat [Nelson & Merckel (112)]. Surprisingly enough, this effect was not only evident but even enhanced after hypophysectomy. In view of the fact that isodehydroandrosterone failed to induce vaginal cornification in the spayed animal, it is suggested that these compounds may be converted to oestrogens in the ovary. The observation of Hohlweg (114) that isodehydroandrosterone, but not androsterone and testosterone, is capable of inducing oestrus in the infantile rat, should be quoted in this connection. This author interprets this effect, as well as the luteinization of the ovary which he noted after administration of isodehydroandrosterone and testosterone, as resulting from stimulation of the pituitary to secrete gonadotropic hormones. In view of Nelson & Merckel's experience with the hypophysectomized rat, it may be doubted that the oestrogenic effect of isodehydroandrosterone is mediated by the pituitary. Evidence that testosterone and isodehydroandrosterone actually suppress the secretion of gonadotropic hormone from the pituitary is presented by Hertz & Meyer (116), who found that the administration of these androgens to the gonadectomized partner of a parabiotic pair of rats prevented the ovarian hypertrophy which otherwise obtains in the normal partner. That the oestrus-inhibitory action of testosterone in the normal cyclic animal is not entirely dependent on this mechanism, but must be, at least partly, due to local action on the vaginal epithelium, may be deduced from the observations of Robson (117) and of Courrier & Cohen-Solal (118) on the antioestrogenic effect of testosterone in oestrin-treated, ovariectomized mice and rats.

The observation of Dempsey (119) that the administration of testosterone or androsterone benzoate to normal female guinea pigs had no effect on the length of the cycle, nor on growth and luteinization of the follicle, indicates that species differences exist in regard to the action of androgens on the female sex tract.

While testosterone and other androgens stimulate development of the rudimentary prostatic elements (Skene's duct) in the female rat to structures resembling the male prostate (120, 121), the enlargement of the prostate of the immature male rhesus monkey induced by oestrone is inhibited by testosterone (122). In male mice, where oestrin treatment actually decreases the weight of the gland by suppressing its secretion, testosterone effects a weight increase of the whole gland, but only corrects the oestrin-induced metaplasia of the

glandular epithelium without reversing the hypertrophy of the fibromuscular stroma (123).

Nelson & Gallagher (107) showed that castration changes in the pituitary of the ovariectomized rat could be prevented by treatment with androstanediol, androstenedione and androsterone. Wolfe & Hamilton (124) point out that the response of the castrate pituitary to testosterone esters differs from that obtained by oestrin treatment insofar as the androgen does not increase the weight of the gland, and induces degranulation of the basophiles only. This difference in action may even manifest itself as antagonism, since testosterone acetate, when administered simultaneously with oestrone to immature female rats, partially prevented the typical oestrin effect: weight increase, and complete degranulation of the basophiles. In the castrated male and female, simultaneous injection of androgen and oestrogen failed to preserve the normal structure of the pituitary, which either of these hormones will maintain when given alone.

Testosterone, unlike oestrone, does not seem to interfere with the output of growth hormone from the hypophysis, since McEuen, Selye & Collip (111) did not observe any inhibition of somatic growth in young rats treated with doses of testosterone sufficient to inhibit gonadal development in either sex and the oestrus cycle in the female. The stimulation of mammary growth and development observed in the male rat during treatment with testosterone (107, 125) is definitely dependent on the presence of the pituitary, since it does not occur after hypophysectomy (108). Robson's report (126) that testosterone propionate (but not androsterone) causes inhibition of milk secretion in lactating mice, evident in deficient growth and limited survival of the litters, is somewhat difficult to harmonize with the findings of Selye *et al.* (125) that testosterone benzoate causes both mammary development and secretion in the male and female rat.

The progesterone-like action of testosterone, apparent in its (weak) progestational effect on the rabbit endometrium [Klein & Parkes (181)], manifests itself, according to Leonard *et al.* (127), also in its capacity to suppress, *in vivo*, the spontaneous oestrus motility of the rabbit myometrium. Hartman (128) concludes that testosterone resembles progestin in that it inhibits, in the cyclic monkey, the onset of menstruation immediately following castration or cessation of oestrin treatment. The inhibiting effects were tried, however, only during the oestrin phase of the cycle. Continuation of oestrin treatment would also have prevented menstruation. These experi-

ments, therefore, do not definitely show a similarity of testosterone and progestin action. In regard to sex-skin stimulation Hartman found that testosterone resembled oestrin, not progestin. Zuckerman (129) reports that testosterone inhibited menstruation in the monkey, but no gestational changes in the endometrium occurred.

OVARY

Oestrogens.— β -oestradiol, the C_{17} epimer of the (α)-oestradiol occurring in sow's ovaries, has been obtained as a by-product in the reduction of oestrone (130, 131). It has also been isolated from the urine of pregnant mares (132). Its oestrogenic potency is only about one-tenth that of oestrone. α - and β -oestradiol can be separated by means of digitonin, only the former being precipitable by this reagent (130). Surprisingly large amounts of α -oestradiol have been isolated from pregnant mare's urine by van Stolk & Lenchère (133). These authors estimated that in some batches of urine this highly potent diol accounts for one-half to one-third of the total oestrogenic activity. The two epimeric 17-dihydroequilenins were prepared by Marker *et al.* (134) by reduction of equilenin. The lower melting of the two diols is identical with the dihydroequilenin occurring in mare's pregnancy urine (135).

Remesov (136) obtained from neorgosterol, by a not very clearly described procedure of oxidative degradation (lacking in characterization of the intermediary products), a non-phenolic isomer of oestrone in which ring B instead of ring A is aromatic. The compound is said to be as potent physiologically as oestrone. Remesov's result does by no means invalidate, as the author contends, the report of Marker *et al.* [*Ann. Rev. Biochem.*, **6**, 154 (1937)] on the preparation of oestrone from neodehydroergosterol. However, the more serious objection of Windaus & Deppe (137) concerning the structure of one of the intermediary compounds in Marker's procedure has not yet been met by these authors. A bond isomer of equilin, isoequilin, has been prepared by Inhoffen (138) from dibromoandrostenedione. The double bond in ring B of this compound is assumed to be either in positions 6-7, or 8-9, instead of in position 7-8 as in equilin. Its oestrogenic potency is much lower than that of equilin (minimum effective dose 50 μ g., method not stated).

The search for derivatives of oestrogens with intensified physiological action has led to the preparation of new esters of oestrone and

oestradiol. Dirscherl (139) reports that the chloroformiate of oestrone is ten times as potent as the free oestrogen. Miescher & Scholz (140) describe the preparation of a large number of new esters of oestrone and α -oestradiol, including those of the higher fatty acids. They accomplished for the first time the preparation of various 17-monoesters and mixed diesters of α -oestradiol. The biological results are briefly indicated in a preliminary note (141). The strongly protracted action of the esters is emphasized. Parkes (142) determines the duration of action of various esters of oestrone, α -oestradiol and oestriol by measuring the width of the brown bar which appears on the black feathers of the Brown Leghorn capon at the site of a single injection.

It would be too far afield to deal here *in extenso* with the numerous reports on synthetic polycyclic compounds exhibiting oestrogenic activity. It might suffice to mention that certain hydroxy compounds (143, 144) and hydrocarbons (145) of comparatively simple structure have been added recently to the list of synthetic oestrogenic agents, although their potency is of a lower order of magnitude than that of the most active members of the dibenzanthracene series. Among the hydrocarbons, triphenylethylene is the most efficient, 5 mg. doses causing oestrus in 50 per cent of the injected animals. The report of Dodds & Lawson (144) that the potency of *p*-propenylphenol equalled that of oestrone was later retracted by the authors (146). The high potency of their original preparation was found to be due to a yet unidentified impurity, although the compound undoubtedly possesses some oestrogenic activity of its own. In preparing the same compound by a different method Serini & Steinruck (147) obtained side fractions, obviously polymerization products, from which after acetylation a crystalline substance, $C_{26}H_{34}O_4$, of high oestrogenic potency (1 Allen-Doisy unit in 5 to 10 μ g.) could be isolated. It is interesting to note that oestrin effects, other than vaginal stimulation, can be elicited by synthetic compounds. Among these are: proliferation of the uterine endometrium (148); prevention of castration changes in the pituitary (149); restitution of mating instincts (150).

Cohen, Marrian & Odell (151) describe an improved procedure for the isolation, from human pregnancy urine, of oestriolglucuronide, which they have now obtained in crystalline form as the sodium salt. Final proof that the phenolic hydroxyl in this compound is free was adduced by methylation and subsequent hydrolysis, which yielded the

3-monomethyl ether of oestriol. The potency of the conjugated oestrogen, computed on its oestriol content, was found to be approximately the same as that of oestriol, when both preparations were given orally; but on subcutaneous injection the action of the glucuronide is much inferior to that of the free oestrogen, which given by this route is about ten times more effective than when given by mouth (152). The comparatively high potency of the glucuronide by the oral route was found to be due to the presence, in the intestines of the test animal (mouse), of a glucuronidase capable of liberating the free oestrogen. Schachter & Marrian (153) obtained evidence that the conjugated oestrogens in mare's pregnancy urine are not glucuronides but sulfuric acid esters. In contrast to findings in the human, no marked changes in the amount of excreted free oestrogen seem to occur in this species during the last months of pregnancy, and at parturition. Mühlbock (154) found that the oestrogen content of the serum of mares during pregnancy parallels the amounts of oestrogen excreted in the urine, attaining a maximum between the sixth and ninth months (2500 to 10,000 I.U. per liter). The oestrogen is present in the serum in a form insoluble in organic solvents.

Westerfield & Doisy (155) determined, by the use of Girard's ketone reagent, the relative amounts of ketonic and non-ketonic oestrogens present in sow's and cow's ovaries, and in human placenta. Only in the latter organ was an appreciable part (25 per cent) of the activity found to be due to ketones; some ketonic oestrogen (5 per cent of the activity) is present in sow's ovaries, while in cow's ovaries such could not be demonstrated with certainty.

Schmulovitz & Wylie (156) describe a colorimetric method for the determination of oestrone based on the production of a red dye on coupling with *p*-diazobenzenesulfonic acid. Venning *et al.* (157) use a quantitative method based on the Kober reaction with phenol-sulfuric acid, in which the error caused by interfering urinary chromogens is eliminated by measurement of the color intensity in two suitable regions of the spectrum. Pincus *et al.* (158) reinvestigated the applicability of the Kober test to the determination of oestrone and oestriol in human and rabbit urine. In later work Pincus & Zahl (159) succeeded in freeing rabbit urine from substances interfering with the colorimetric determinations by somewhat modifying the fractionation procedure of Cohen & Marrian. The Kober and Schmulovitz-Wylie reactions can thus be used for the assay of oestrone and oestriol in the respective fractions, and oestriol can, in

addition, be determined by the specific reaction of David with sulfuric and arsenic acid. The method was applied to the study of oestrone and oestriol excretion in the female rabbit after injection of these hormones. Animals in oestrous, pseudopregnant, pregnant, hysterectomized and ovariectomized conditions were used. The results indicate: (a) that while oestriol is excreted unchanged, injected oestrone is partly converted to oestriol; (b) that this conversion requires the presence of a functional uterus and probably occurs under the influence of progesterone; (c) that luteal secretion prevents to a certain extent the destruction of the injected hormones. Westerfield & Doisy (155), working with the monkey, also observed partial conversion of injected oestrone into a non-ketonic form, while α -oestradiol was found to be excreted partly in the form of a ketonic oestrogen. However, these conversions also occurred in the ovariectomized hysterectomized animal.

The studies of Koch and his collaborators (101, 102) on the daily oestrogen excretion of normal subjects, and in certain pathological cases, have been mentioned in the section on androgens. The two peaks in the excretion of oestrogen by normal females at mid-interval, and shortly before menstruation, are more clearly brought out in the data of Yerby (160), who, however, omitted hydrolysis of the urine. Palmer (161) differentiated in his excretion studies between free and combined urinary oestrogen. It would appear from his excretion curve that oestrogen is excreted during the intermenstruum only in the combined form except for the sudden appearance of large amounts of free oestrogen at the mid-interval and a slight increase before the onset of bleeding. The mid-interval rise of free oestrogen is thought to be caused by the sudden peritoneal absorption of follicular fluid from the ruptured follicle and excretion in the urine before conjugation can occur.

Curtis, Miller & Witt (162) report that ovariectomized rats may acquire marked tolerance to oestriol on prolonged treatment, so that doses fifty times or more the initial dose have to be used to produce the vaginal reaction, although the sensitivity to oestrone does not change during that period.

Freed, Garvin & Soskin (163) believe that the difference in the uterine response of the normal and of the ovariectomized female rat to prolonged treatment with α -oestradiol benzoate indicates the existence of an endocrine factor other than oestrin or progestin in the normal ovary. This hypothesis is further elaborated by Freed &

Soskin (164) on the basis of experiments with immature female rats which were treated at ages varying between six and twenty-one days with pregnancy-urine extract. At an early age this treatment resulted in luteinization of the theca and interstitial cells only, while the uterine endometrium remained infantile. At a later age (seventeen to twenty days), when the ovary responded to the injections with follicular growth and luteinization of the granulosa, full uterine oestrus was observed. This is taken to indicate the existence of two different oestrogenic hormones, one arising in the theca and the other in the granulosa. The question may be raised whether such a far-reaching assumption is necessary to explain the experimental facts. The observed differences in the state of the endometrium may be due to a low oestrin output from the theca, insufficient to call forth uterine oestrus, and a subsequent rise above the threshold value when follicular growth sets in.

The view that the beneficial effect of oestrin treatment in the gonococcal vaginitis of children is due to a decrease in the acidity of the vaginal contents (165, 166) is not borne out by the measurements of Ranson & Zuckerman (167) who found that oestrone treatment had no effect on the rather variable vaginal reaction of the monkey. In van Dyke & Ch'en's (168) experiments on the immature monkey, however, a definite rise of vaginal pH after ovariectomy was observed, which could be counteracted by treatment with oestrin. The coincident changes in the glycogen content of the vaginal mucosa (decrease after ovariectomy and rise after oestrin) point to lactic acid as being concerned in this phenomenon.

Kun & Pecsénik (169) showed that the minimum dose of testosterone and of other androgens, required for inducing ejaculation in the castrated rat on electrical stimulation, could be decreased many times when oestrone was administered simultaneously, although the latter hormone was ineffective when given alone.

Dempsey (119) could not detect any inhibition of follicular growth in guinea pigs, from which the corpora lutea had been removed, after daily injections with 250 I.U. of oestradiol benzoate for ten to twenty days, although the treatment interfered with ovulation by inducing atresia in the last stage of development. Similar results were obtained by Emery (170) in the female rat. Injection of two to twenty rat units of oestrone daily for six weeks into normal animals did not affect the size of the ovaries or the length of the oestrus cycle, and did not prevent normal hypertrophy of the remaining ovary in

animals unilaterally ovariectomized. Furthermore, this treatment did not interfere with the normal regeneration of ovarian fragments left *in situ* after partial bilateral ovariectomy. Such results are difficult to reconcile with the bulk of evidence indicating that high levels of oestrogen, maintained for a sufficient period of time, inhibit the output of gonadotropic factors from the pituitary. That such an inhibition can be produced with small amounts of oestrogenic hormone is evident from the extensive experiments of Meyer & Hertz (171). These workers employ the ovarian hypertrophy, obtained in the immature female rat put into parabiosis with a castrated male or female litter mate, as an index of the level of gonadotropic secretion from the pituitary of the latter. The administration of very small amounts of oestrone (0.2 to 1 μ g. per day) to the gonadectomized partner completely inhibited the ovarian hypertrophy in the normal parabiont, indicating suppression of gonadotropic secretion. Hohlweg & Chamorro (172) found that the luteinization of the ovaries of immature rats (eight weeks old) induced by a single dose (11 μ g.) of α -oestradiol benzoate was first noticeable five days after injection. Hypophysectomy on the second day after the injection prevented the effect, but failed to do so after a lapse of four days. From this it is inferred that liberation of luteinizing hormone from the pituitary occurred between the second and fourth day following oestrin administration, e.g., about simultaneously with oestrus.

That large doses of oestrin inhibit lactation in postpartum rats and decrease the amount of lactogenic hormone in their pituitaries, as compared with that of uninjected controls, was ascertained by Wiegand (173).

Victor & Andersen (174) showed that oestrone or α -oestradiol, added to anterior pituitaries of spayed rats *in vitro*, increased the oxygen consumption of this tissue. The anterior pituitaries of similar animals injected with these hormones likewise showed increased respiratory rates within six hours after administration. The effect appears to be specific for this tissue, since kidney and liver slices did not respond in the same manner. Since it had been found previously that the respiratory rate of the rat pituitary is higher in pro-oestrus than in any other phase of the cycle (175), and that it also rises during parturition (176), it seems possible, in the opinion of the authors, that the phenomenon is related to the liberation of gonadotropic hormone from the gland as a result of oestrin stimulation.

Corpus luteum.—The isolation of pregnenolone from the chromic

acid oxidation products of cholesterol acetate dibromide (177), and the synthesis of the same compound from isodehydroandrosterone (178) would seem to open new avenues for the preparation of the corpus luteum hormone from cholesterol. Westphal & Schmidt-Thomé (179) prepared the Δ^5 -bond isomer of progesterone, and found it lacking in progestational activity. Hence, the organism is apparently not able to effect the rearrangement to Δ^4 -progesterone which takes place with extreme ease *in vitro*. The enol esters of Δ^4 -progesterone, prepared by Westphal (180), which possess probably the $\Delta^{3:5}$ -structure, owe their physiological activity undoubtedly to rapid hydrolysis in the tissues, with concomitant rearrangement, to Δ^4 -progesterone. Klein & Parkes (181) showed that the high degree of specificity generally accorded to the progesterone structure holds only to a limited extent, since various androgens, especially those substituted with a methyl or ethyl group at position 17, exhibit some progestational activity when given in sufficient doses.

Allen & Goetsch (182) and Butenandt & Westphal (183) described improvements in the preparation of progesterone from corpus luteum extracts.

It is now evident that pregnanediol and *allopregnanediol* are not the only reduction products of progesterone which are excreted by the human during pregnancy. From 10,000 gallons of pregnancy urine Marker, Kamm & McGrew (184) obtained a few grams of a new hydroxyketone, *epi-allopregnan-3-ol-20-one*, the C_8 -epimer of the *allopregnanolone* present in corpus luteum extracts. In a subsequent publication Marker *et al.* (185) describe the preparation of the new compound from 3-chloro-*allocholanic* acid, and report the remarkable fact that it exhibits androgenic activity about equal to that of androsterone in the rat seminal vesicle test. Later, *epipregnan-3-ol-20-one*, the C_8 -epimer of *epi-allopregnanolone*, was isolated by Marker & Kamm (186) from the same source. That the excretion of pregnane derivatives during pregnancy is not confined to the human has been shown by Marker *et al.* (187), who succeeded in isolating pregnanediol from pregnant mare's urine. The presence of *allopregnanediol* was ascertained by the isolation of *allopregnanedione* from the carbinol fraction after treatment with chromic acid.

The occurrence in human pregnancy urine of an ether-insoluble conjugated form of pregnanediol was demonstrated by Odell & Marrian (188) and Venning & Browne (189). The latter investigators succeeded in isolating the conjugated compound in crystalline form

and identified it as the sodium salt of pregnanediol glucuronide. The method of isolation is so simple that it permits the preparation of pregnanediol in excellent yields; moreover, it can be easily adapted to quantitative studies of pregnanediol excretion (190). From the data published by Venning & Browne it would appear that the quantitative method is to become an important tool for determining the functional activity and duration of the corpus luteum. The assumption that the excretion level of pregnanediol glucuronide is a measure of the amount of progesterone secreted by the functional gland is borne out by the following observations: The compound is excreted only during the luteal phase of the cycle; it appears in the urine as far as can be ascertained shortly after ovulation and disappears again from one to three days before the onset of menstrual bleeding (191); it is found in the urine after intramuscular injection of progesterone (190). During the first two to three months of pregnancy the excretion remains on the level found during the luteal phase of the cycle, then rises until a maximum is attained in the eighth month, and falls abruptly before parturition (192). The observation, in one case, that the excretion of the compound persisted after removal of the ovary carrying the corpus luteum may indicate that the placenta can participate in the production of progesterone.

The ease with which reduction of progesterone to pregnanediol seems to take place in the human organism is perhaps the explanation for the comparatively low progestin content of human corpora (193) and its absence from the blood (194) and urine of pregnancy. On the other hand, the corpus luteum of the pregnant mare, which also excretes pregnanediol, is rich in progestin (195).

Pincus & Werthessen (196) try to obtain greater precision in the rabbit-assay method by measuring the size of the tubular ova, and, planimetrically, the ratio of uterine glandular area to total mucosa area as indices of progestin activity. Christensen (197), using the Clauberg technique, measures the percentage of positive (submaximal) reactions in two groups of animals receiving the unknown and a standard preparation, respectively. Comparison of the relative strength of the two preparations is then made by means of a previously determined percentage-reaction-dosage curve. Hertz, Meyer & Spielman (198) make use of the apparently specific capacity of progesterone, first noted by Dempsey, Hertz & Young (199), to elicit the copulatory reflex in oestrone-conditioned, ovariectomized guinea pigs, to detect small amounts (0.05 I.U.) of the hormone.

The hormonal factors involved in the mucification of the vaginal epithelium in ovariectomized rodents was investigated anew by Selye, Browne & Collip (200) in the rat, and by Klein (201) in the golden hamster. The effect is attributed to a synergism of balanced amounts of oestrone and progesterone. The amount of progesterone necessary to counteract the oestrogenic action of a given dose of oestrone or α -oestradiol in the ovariectomized mouse and rat was determined by Robson (117) and Courrier & Cohen-Solal (202). That sufficiently large doses of progesterone suppress the normal oestrus cycle of rodents was ascertained by Robson (117) in the mouse, and by Phillips (203) and Selye *et al.* (204) in the rat. The latter investigators ascribe this to the ovarian atrophy observed in the progesterone-treated animals. Makepeace *et al.* (205) inhibited ovulation in the post-coital rabbit by injection of progesterone. Since ovulation took place in progesterone-treated animals after injection of a gonadotropic preparation, it is assumed that the site of inhibition is not the ovarian follicle, but that progesterone interferes with the output of gonad-stimulating hormone from the hypophysis. The same idea is advanced by Dempsey (119) on the basis of analogous findings in the guinea pig. The finding of Zwarenstein (206) that injection of progesterone can induce ovulation in the South African clawed frog, even after hypophysectomy, is without parallel in mammalian physiology, but Shapiro (207) demonstrated that the effect is unspecific and can be elicited by various androgens and a few other steroids.

The results of Robson (208) show that progesterone can replace the lost luteal function in hypophysectomized rabbits insofar as it maintains gestation for varying periods of time after the removal of the pituitary. This is true for animals hypophysectomized in the early as well as in the late stage of pregnancy. In no single experiment, however, was gestation carried to term. The same author (209) reports the somewhat surprising fact that the structure and function of the corpus luteum itself can be maintained in the hypophysectomized pseudopregnant rabbit by the administration of crystalline oestrogens. It is suggested that the control exerted by the pituitary over the corpus luteum may be indirect, through the production of oestrin. Reynolds, Firor & Allen (210), confirming an older observation of Robson, find that more progesterone (four rabbit units) is required to evoke progestational changes in the endometrium, and to inhibit the oestrin-induced motility of the myometrium, in hypophysectomized rabbits than in rabbits with the pituitary present.

Menstrual cycle.—Hisaw *et al.* (211) have reported further on the uterine and other effects of combined treatments with oestrin and progesterone in spayed sub-adult monkeys. Following a preliminary oestrin treatment, the combined injections caused a much greater increase in uterine size than did progesterone alone. This increase involved both the endo- and myometrium. The basal portions of the uterine glands showed a much greater distension and finally reached a stage of secretory exhaustion. The antagonism between oestrin and progesterone which has been reported from work on rabbits was not exhibited by the monkey within the range of dosages used (100 R.U. + 4 Rb. U. to 300 R. U. + 1 Rb. U.). In rabbits, a much smaller oestrin-progestin ratio [10 R. U. to 1 Rb. U. (212); 1/75 weight of progestin, i.e., about 100 I. U. or 20 R. U. to .75 Rb. U. (213)] inhibits the effect of the progestin, although W. Allen has shown that a small amount of oestrin added to progestin increases the duration of the pro gravid stage. Zuckerman (214) found a well-developed progestational endometrium in a spayed monkey after treatment with 150 I. U. oestrone + 1 Rb. U. progestin, but in another experiment (No. 4) states that 300 I. U. oestrone inhibited 5 mg. (5 I. U. or 5 Corner-Allen Rb. U.) progesterone. The illustration of the endometrium, however, appears to show some progestational changes and the author states that the glands are somewhat dilated. It is clear (211) that in monkeys a lower oestrin-progestin ratio will cause a disappearance of sex-skin response, metaplasia of the cervical epithelium, and vaginal cornification than will negate the progestin effect on the endometrium.

Zuckerman (214, 215, 216) has ascertained the daily dosage of oestrone which, given over long periods to spayed monkeys, will result in periodic bleeding or in continued amenorrhea. He finds that when 100 I.U. are given daily, uterine bleeding will take place every five to seven weeks. This periodic bleeding he attributes to a change in the oestrin threshold of the endometrium. Dosage well above this threshold, when given for long periods, will not induce bleeding until the treatment is stopped. It has been suggested by Long & Zuckerman (217) that the periodic bleeding with threshold dosages is due to a coincident cycle in the activity of the adrenal cortex and the attendant fluctuations in water and salt metabolism. The basis for this hypothesis is supplied by studies on water metabolism during normal cycles in a mature monkey (218). Water retention in the first half of the cycle and water loss in the second half were observed, these

changes paralleling the swelling and subsidence of the sexual skin. van Dyke & Ch'en (168) found that the endometrium contained more water during the proliferative stage than during menstruation. Zuckerman (214) also repeated the earlier experiments of Smith *et al.* (219), Engle *et al.* (220), and Hisaw (221) in which it was shown that in castrated monkeys primed with oestrin, progestin injections will inhibit the uterine bleeding which would have otherwise resulted from the oestrin withdrawal. He has also confirmed the work of the above investigators who showed that bleeding will follow the withdrawal of progesterone even though oestrone injections are instituted. He states (214) that a series of progesterone injections for periods of ten to twelve days in the latter half of the cycle, days twelve to twenty-six, does not interfere with the length of the cycle and uses this and other evidence for drawing the conclusion that "a wave of estrogenic stimulation must be the basic mechanism of the endometrial cycle." In addition to the work quoted above, which has shown in spayed monkeys that the oestrin control of a complete cycle is superseded by a progesterone control, and that oestrone is then ineffective in substituting for progesterone, it has been shown by Corner (222) in intact monkeys that progesterone administration begun in the last half (luteal phase) of the cycle will delay the next expected menstruation for long periods. The next menstrual cycle was shifted by the time interval corresponding to the delay. Oestrone injections begun in the luteal phase in the normal animals did not inhibit the next menstruation. If, as postulated by Long & Zuckerman (217), a periodic fluctuation in cortical activity plays a rôle in the cycle, the progesterone injections must have changed the periods of this fluctuation. The assays of the oestrogen content of the blood reported by Frank (223, and earlier) and Fluhmann (224) show that for a few days preceding menstruation the oestrin in the blood is high [25 M.U./liter (223), 60 M.U./liter (224)], roughly 150 to 360 I.U. of oestrone constantly in the circulation. Clinical tests have also shown that the administration of progesterone but not of oestrone, when begun in the luteal phase of the cycle, will inhibit menstruation (225). The work of Venning & Browne (191) which showed that pregnanediol abruptly disappeared from the urine one to three days before the onset of menstruation supports the concept that menstruation in the ovulatory cycle is due to progestin withdrawal. The characteristic endometrial responses can be induced in hypophysectomized monkeys by oestrone and progesterone injections, and the time of

menstruation or withdrawal of treatment is not significantly altered although the adrenals show a very pronounced atrophy (226). The concept, which is still being maintained by some investigators, that oestrone is the only important hormone in the menstrual cycle arose from the earlier studies of E. Allen on oestrone treatments in spayed monkeys and from the fact that monkeys in captivity show periodic uterine bleeding although ovulation and corpus luteum formation do not take place. This type of cycle, which would never permit the propagation of the species, involves only the action of oestrone. In the type of cycle which is essential for fertility, the corpus luteum secretion becomes of predominant importance in the second half of the cycle, and its withdrawal terminates the cycle and is the immediate cause of menstruation. The complete (ovulatory) cycle thus involves the action of both hormones and it consequently cannot be stated that either one is predominant in importance. The fact that progesterone without previous oestrin injections will induce a response of a luteal character (227) or that oestrone increases the action of progesterone on the endometrium (211) does not vitiate the concept of the oestrone-progesterone relationship in the ovulatory cycle. Nor does the finding that testosterone administration, after withdrawal of oestrone treatments (128, 129), prevents bleeding, void this relationship for the endometrium does not show prograavid (luteal) changes with testosterone treatments (129).

Certain procedures have been developed which will further aid in the determination of the time of ovulation in women. Rubenstein (228) in a large number of cycles recorded, daily, the type of vaginal smear and body temperature. He found a characteristic drop in temperature at the time when the ovulative type of smear appeared. There was much variability in the temperature readings, however, and the drop was not sufficient to be definitely recognizable until all the data for a cycle were available. Burr *et al.* (229) in one case and Rock *et al.* (230) in one case, recorded in women the difference in electrical potential between the vagina and abdominal wall or symphysis at about the mid-interval. Following a marked change in potential, laparotomies were performed and in each case a freshly ruptured follicle was found. In the one case in which data were given (230) ovulation occurred on day fourteen of the cycle and the endometrium was in the proliferative stage. Finch *et al.* (231) took daily records through two cycles in a chimpanzee, the electrodes being placed on the back of the hands. In each cycle a maximum

change in potential appeared on day seventeen. At about this time the genital swelling decreased. In the next cycle a second peak in potential occurred which, because of a subsequent positive Friedman pregnancy test, was thought to be correlated with fertilization and early pregnancy changes. The occurrence of ovulation was not checked by laparotomy. This is an application to the human and primates of the method used by Burr *et al.* (232) and Reboul *et al.* (233) on rabbits.

ANTERIOR HYPOPHYSIS

Gonadotropic hormones.—Greep & Fevold (234) have reported experiments with their preparations of follicle-stimulating (FSH) and luteinizing (LH) fractions of the hypophysis in adult male hypophysectomized rats. Their results are in general agreement with those reported earlier (235) on immature rats. In treatments with FSH begun immediately after ablation of the hypophysis, there was no interruption of sperm formation for the duration of the treatment (forty days). The accessory sex structures underwent early regression. Injections of LH maintained spermatogenesis and the accessories, although the accessories but not the tubules showed some regression after thirty to forty days. When fifteen days elapsed between hypophysectomy and the beginning of treatment, the injection of FSH restored spermatogenesis but had only a slight effect on the accessories. Injection of LH restored both the gametogenic and endocrine functions of the testes. The addition of testosterone to FSH did not enhance the spermatogenic function of the latter extract. The one difference between the work on the mature and the immature rat is that LH in the latter had no effect on the epithelium of the seminiferous tubules.

Cutuly *et al.* (236, 237) have reported an interesting series of parabioses in rats. They united hypophysectomized males to castrated males. The testes and the accessory reproductive organs of the hypophysectomized member of the pairs were completely maintained. The significant feature of this experiment appears to be the maintenance of the accessories as well as the testes. This finding is difficult to harmonize with the current concept that FSH stimulates only the seminiferous tubules and that LH maintains the accessories through the stimulation of the interstitial tissue. It has been shown in a number of papers that in hypophysectomized-female castrated-male pairs, the ovaries show only follicle development. This has been used as

evidence that the gonadotropic hormone liberated by the hypophysis of a castrated male is FSH as have the findings that ovaries grafted into a castrated male (238) show only follicular development. If it be true that the hypophysis of the castrated male rat liberates only FSH and that FSH causes only seminiferous tubule development, then in the pairs of Cutuly *et al.* the hypophysectomized member should not have shown any maintenance of the accessory reproductive organs. The situation in regard to the gonadotropic hormones becomes more complex with an increase in the type of tests. Four hormones affecting gonad function (as follicle stimulator, luteinizer, interstitial cell stimulator, and antagonist) are reported by Evans *et al.* (239, 240). It seems not improbable that results may be brought more into harmony when the effect of such factors as dosage, length of treatment, and rate of absorption have been more fully explored.

Cole (241) has reported some very striking results from injections of the gonadotropic substance of pregnant mare serum into mature and immature female rats. A single injection of 12 R.U. given in the metoestrus decisively increased the number of young in a litter. More pronounced effects, however, were found with immature animals. He secured as many as 28 implantations and the average number equaled that in mature animals. Of 30 which mated, 43 per cent had 17 or more foetuses, fifteen to seventeen days after mating, and as many as 17 living foetuses weighing but slightly below normal, were born to these small mothers. The uteri, when unfolded, exceeded the body length of the mother. Dating from 1927 when Smith & Engle reported the liberation of as many as 70 normal ova in precociously matured mice, and Engle secured a large number of implantations, several investigators have secured ovulation from injection of gonadotropic hormone in animals which would not normally have ovulated. The large number of foetuses brought to term in the experiment of Cole reveals an unexpected potential capacity of the uterus as well as ovarian effects which may be secured when proper dosages are used. Davis (242) states that in half of 50 women given a single injection of an extract of pregnant mare serum, ovulation occurred, as revealed by laparotomies performed eighteen to thirty-six hours after the injection. Ovulation was determined by the presence of corpora lutea and not by the finding of ova in the tubes.

Cartland & Nelson (243) describe a method for preparing highly active gonadotropic extracts from the blood plasma of pregnant

mares based on fractional precipitation with acetone at properly adjusted acidities. The procedure has the advantage over other precipitation methods in that most of the inert protein is removed by fractionation steps which leave the active principle almost quantitatively in the supernatant fluid. Yields of 60 to 90 per cent of the total activity, with 130-fold increase in potency, are reported. Further precipitation yielded fractions 1800 times more potent than the original plasma.

The extensive literature about the augmentation of response when various non-gonadotropic substances are added to certain gonadotropic preparations has been added to by the discovery that haeme will cause augmentation. McShan & Meyer (244), by fractionating erythrocytes, find that augmentation from the formed elements of blood, which had been observed earlier by Cassida and by Kraats, is due to the haeme, not to the globin fraction. The same amount of iron contained in the haeme, when added to the gonadotropic extract, did not enhance its effect. Although the haeme had to be added to the extract in order to get augmentation, they feel that the enhancing action is not due to a slowing of absorption since globin caused a precipitate when added to the extract but did not give augmentation. It is obvious that the augmentative effect of blood cells (and of serum, also) makes determinations of the gonadotropic content of blood, by means of the augmentation reaction, of questionable validity. Thompson (245) has described a type of augmentation which appears to be of a different nature from those described by other investigators. After a series of injections of sheep hypophyseal extract, prepared by the method of Wallen-Lawrence & van Dyke, he found that the serum, for a variable period, augmented the weak gonadotropic action of the extract even when the two were injected in opposite sides of immature female rats. Normal serum did not have this action, nor did the immune sera when injected alone into hypophysectomized rats, although it had a gonadotropic action in normal animals. This augmentative action is attributed to an anti-substance developed in response to a gonad antagonist contained in the injected extract.

Fevold *et al.* (246) believe that the augmentation of gonadotropic extracts by copper is of a different nature from that given by zinc salts. In normal rats, copper augmented the action of FSH and of FSH + LH, but in hypophysectomized rats the action of FSH was not augmented by copper, although the response elicited by the com-

bined fractions was increased when copper was added. They attribute the augmentative action of zinc to a slowing of absorption, as do other investigators. Copper, they believe, gives augmentation by catalyzing the synergistic action between FSH and LH. They report, as additional evidence in support of this hypothesis, that copper salts cause ovulation in rabbits. A considerable number of other "non-gonadotropic" substances induce ovulation in rabbits. Pfeiffer (247) finds in rats that the injection of copper sulphate has a toxic effect but has no action on the endogenous hormones. The action of injected luteinizing hormone, however, was augmented by being mixed with copper sulphate.

Lactogenic hormone.—Lyons (248) gives detailed directions for the preparation of highly potent lactogenic fractions from sheep pituitary. An important factor of the method is the elimination of adrenotropic activity, which is accomplished by isoelectric precipitation at pH 6.5; the lactogenic hormone remaining in the supernatant fluid is then precipitated at pH 5.5. The final product was free from gonadotropic and thyrotropic activity and contained very little adrenotropic hormone. White, Catchpole & Long (249) succeeded in obtaining a crystalline preparation of approximately the same lactogenic potency by a technique similar to that employed in the crystallization of insulin. The product gives the usual protein reactions. The authors point out, however, that the positive outcome of the Millon, xanthoproteic and labile-sulphur tests is difficult to reconcile with Evans' statement (250) that his most potent lactogenic preparations did not give these reactions. Bergman & Turner (251) compared the efficiency of various methods for extracting the lactogenic activity from sheep glands and give preference to the use of dilute alkaline alcohol.

Gomez & Turner (252), in an extensive paper covering their recent work, report that although daily implants of normal rat hypophyses into immature hypophysectomized guinea pigs caused no mammary duct development, implants would stimulate development if the donors of the implants had been injected with 100 I.U. of oestrogen daily for ten to twenty days. It will be recalled that Selye *et al.* (253) found that implants of normal hypophyses into hypophysectomized rats would not maintain lactation. This finding of Gomez & Turner is one of a number of experiments (252) which lead them to postulate a "mammary growth-stimulating hormone." In hypophysectomized guinea pigs various hormones (oestrogen, progesterone,

cortin, adrenotropic, thyrotropic) given singly or in combinations to hypophysectomized animals, did not produce mammary gland development, although after partial hypophysectomy oestrin injections caused growth of these glands. The injurious effects of adrenalectomy described by a number of investigators have been confirmed by Levenstein (254) in rats. Salt therapy induced normal lactation in some of the animals. McEuen *et al.* (108) find that testosterone (0.2 mg. per day) stimulates mammary gland development in normal male rats but not in hypophysectomized ones. An extensive literature is harmonious in finding that the oestrogens and androgens have no direct effect on the mammary glands but affect the latter through their action on the hypophysis.

Definite progress has been made on the factors responsible for the initiation and maintenance of lactation after hypophysectomy. Gomez & Turner (252), Nelson & Gaunt (255), and Nelson (256) have published further on the findings which were reported in abstract form in 1936 (257, 258). Lactogen will neither initiate nor maintain lactation after hypophysectomy. However, the addition of adrenal cortical or adrenotropic hormone causes lactation. The effect is enhanced by the administration of glucose (252). The thyroid appears to play no important rôle in lactation except as it may influence general metabolic activity (259, 252). In assays in a series of animals, Gomez & Turner (260) have shown that following parturition in rats, the lactogen potency of the hypophysis is increased two-fold over that of the non-pregnant animal. Suckling reduced the lactogen content of the hypophysis to one-third that of animals from which young had been removed for a fifteen-hour period after a preliminary period of nursing. In contrast to the earlier assays by Bates *et al.* (261), Gomez & Turner (260) find that the lactogen content of foetal cattle hypophyses is lower than that of the adult.

Bates *et al.* (262) have quantitatively studied the anti-gonad action of lactogen (prolactin). They find that prolactin represses the gonads by inhibiting the release of FSH from the hypophysis and that the daily output of this hormone in the adult male dove is equivalent to the M.O.D. of a four month-old New Zealand rabbit. Bates *et al.* (263), in hypophysectomized pigeons but not in rats, obtained body growth with rapid and excessive increase in liver weight by injections of prolactin. Schooley *et al.* (264), in the same test animal, report an increase in weight, length, and villus length from prolactin injections. Lyons (248), however, did not secure an increase in body weight in

hypophysectomized rats with daily injections of mammotropin (unheated) with a dosage equivalent to 10 gm. a day of fresh undissected sheep hypophyses.

Hesselberg & Loeb (265) have repeated the studies reported in 1921 by Kuramitsu & Loeb on the effect of ligation of one of the nipples in nursing guinea pigs. They find that suckling of the non-ligated gland does not prevent atrophy of the ligated gland. The rate of atrophy of the latter was characteristic of that of animals whose young had been removed. In this investigation they examined the glands from three to twenty days after ligation, thus meeting the criticism of Selye (266) who found in rats that cutting of the galactophore, thus preventing nursing, did not cause atrophy if the intact glands were nursed. The findings of Loeb are in harmony with those reported by Hammond & Marshall (267) in rabbits. The latter also state that in sows suckling small litters, it is common "to see the glands used by each pigling large and active while the remainder become small and atrophic." With the great species variation revealed by other work on reproduction, a lack of uniformity of response to disuse of some of the mammary glands in nursing animals of different species is not surprising.

ANTIHORMONES

Several reports have appeared on "antihormone" formation in treatments involving homologous species. Thompson (268) reported that there was no anti-gonadotropic substance in the serum of two ewes treated with 25 cc. of an alkaline extract of whole sheep hypophysis, daily, for six months. Sulman (269) and Kinderman & Eichbaum (270) have confirmed the failure of an anti-gonadotropic substance to develop in the serum of women treated with human pregnancy-urine extracts and the latter could find no evidence of the spontaneous appearance of anti-substances during or following pregnancy. The earlier findings of Selye *et al.* (271), that rats given implants of rat hypophyses lose their sensitivity and had ovaries of subnormal size, and of Collip [cf. Marrian & Butler (272)] that sheep injected with gonadotropic extract prepared from sheep hypophyses will passively immunize rats against gonadotropic extracts prepared from glands of several species of animals, are difficult to harmonize with the findings of Thompson (268), of Katzman *et al.* (273) and the earlier work of Smith (274). Both the latter investigators implanted rat hypophyseal tissue into rats for several months and found

that the ovaries of the recipients exceeded normal size (273) and that the testes were normal in size and structure, and matings were fertile (274). Additional long-term parabioses have been reported by Cutuly *et al.* (236) which are in agreement with those of DuShane *et al.* (275) and others in regard to a continued stimulation of the gonads of an hypophysectomized rat by the hypophysis of a parabiotic mate.

Additional reports have also appeared on the development of inhibitory substances in treatments involving heterologous species and on the species specificity of the antihormonal sera. Oudet (277) reported that guinea pigs which had become refractory to a crude beef thyrotropic extract responded to a purified extract made from sheep hypophyses (preglandol). Thompson & Cushing (278) noted that the serum of one of their dogs which had been chronically injected with a plain alkaline extract of sheep hypophyses would not inhibit the effect of a sodium sulphate preparation of sheep glands although the alkaline extract was inhibited. The serum of another dog injected for a longer period inhibited both preparations. Rowlands (279, 280), Thompson & Cushing (278), Thompson (281), Gegerson *et al.* (282), and Zondek *et al.* (283), with injections of extracts over long periods, have conclusively shown that there is considerable crossing of the inhibitory properties of the anti-sera formed by chronic injections of extracts of hypophyses of one species with hypophyseal extracts from another species and with gonadotropic preparations of human pregnancy urine or pregnant mare serum. This is possibly analogous to the species-crossing of the antibody to purified thyroglobulin, as determined by the precipitin reaction (284, 285), although Schulhof (286) was unable to inhibit the B.M.R. stimulating effect of thyroglobulin by anti-thyroglobulin sera of high precipitin titer and Rosen & Marine (287) also found that refractoriness did not develop to the administration of iodothyroglobulin to rabbits for periods of 39 to 84 days. This latter was evidenced by the continued maintenance of a slightly elevated B.M.R., despite the development of a significant precipitin response against the iodothyroglobulin used. It should be noted, however, that Kestner (288), using an extract of pig-thyroid gland, produced an anti-serum in sheep which depressed the B.M.R. of rabbits, dogs, and rats for several weeks. The data concerning species specificity of the anti-sera against hypophyseal hormones is presented in the accompanying table (Table II). Most of the work was done with gonadotropic extracts.

TABLE II
SPECIES SPECIFICITY OF INHIBITORY ACTION OF CHRONIC INJECTIONS OF GONADOTROPIC HORMONE INTO HETEROLOGOUS SPECIES (+, INHIBITION; —, NO INHIBITION)

Author	Species of AP used to pro- duce antisera	Species of AP, urinary or blood extract assayed								
		PU*	Human	Ox	Horse	Sheep	Swine	PM†	Rat	Rabbit
Fuhrmann (304)	Human	+	+			—				
Rowlands (279, 280)	PU*	+	+	—	—	—	—	—		
Gegerson <i>et al.</i> (282)	PU	+	+	+		—		—	—	—
Kabac (300)	PU	+	+	—						
Zondek & Sulman (283)	PU	+	+							
Selye <i>et al.</i> (301)	PU	+								
Rowlands (279, 280)	Ox	±	+	+	+	+		+		+
Gegerson <i>et al.</i> (282)	Ox	—		+			—			
Eichbaum <i>et al.</i> (302)	Ox‡			+						
Rowlands (279, 280)	PM†	—	—	—	—	—	—	+	+	+
Thompson & Cushing (278)	PM	±		—		±	±	+	+	+
Zondek & Sulman (283)	PM	—		—		—		+	+	+
Meyer & Gustus (303)	PM	—	—			—		+	+	+
Rowlands (279, 280)	Horse	±	+		+	—	—	+		
Thompson & Cushing (278)	Sheep	+		—	+	+	+	+		
Thompson (281)	Sheep‡		+	+						
Eichbaum <i>et al.</i> (302)	Swine‡			—			+	+		

* Human pregnancy urine.

† Pregnant mare serum.

‡ This reference summarizes previous work by Rowlands and by Rowlands & Parkes.

§ Thyrotropic extract.

It is evident that the results vary somewhat. The explanation of the discrepancies may perhaps be found in quantitative differences in the potency of the anti-sera, as well as in the method of preparation of the antigenic extracts. The quantitative factor seems to be extremely important. Parkes & Rowlands (289) have investigated class specificity as well as species specificity. Injection into the domestic fowl of a potent ox-hypophysis anti-serum, crossing with the thyrotropic and gonadotropic hormones of several mammalian species, had no effect upon the activity of the avian hypophysis, as judged by the plumage after administration of amounts of serum up to 20 cc.

The investigation of hormones other than those already known to produce refractoriness and anti-substances in serum gave some interesting results. Hartman *et al.* (25) found that purified cortin containing no protein would lose its effect on the normal dog after repeated injections, as seen by the failure of later injections to produce the decrease in sodium and increase in potassium excretion in urine, which occurred with the initial treatments. Toby & Lewis (290), in the same laboratory, found that the serum of the resistant dogs when injected into other dogs would inhibit the effect of cortin. Cortical extracts gave a complement fixation reaction with serum from the refractory but not from normal dogs. Refractoriness to repeated parathyroid-extract administration has been shown by Taylor *et al.* (291) not to be due to any anti-substance in the serum. Their sera failed to give complement fixation or to inhibit the action of parathyroid extract biologically. The refractory animals survived the injection of massive doses of irradiated ergosterol which invariably killed non-tolerant dogs. Brandt & Goldhammer (292) have produced complement-fixing antibodies to an androgen and an oestrogen by injecting them mixed with swine serum. Such anti-sera, however, did not inactivate these hormones physiologically.

The lactogenic and glycotropic (contra-insulin) hormones have received attention from Young (293). He was able to discern no inhibitory properties developing in sera of animals treated daily for 95 days. In some cases the sera gave augmentation. Young (294) has also shown that the administration of increasing doses of hypophyseal extract for approximately four weeks led (in two of three dogs) to a permanent diabetes which persisted until the death of the animal in one case (ten months), and for the duration of the observations in another (two months). Thompson (245) explains the augmentation of the effect of hypophyseal extracts by some anti-sera,

in his experiments, by the development of antihormone to the hypophyseal antagonist described by Evans *et al.* (239). This antihormone, he feels, removes the inhibitory effect of the antagonist in hypophyseal extract, thus accounting for the augmentation obtained in the absence of FSH in the sera.

An investigation of the chemical and physical properties of antihormones has been reported by Zondek & Sulman (295, 296) using human pregnancy-urine gonadotropin. They concentrated the hormone in the globulin fraction of the serum and prepared a powder from this fraction. This powder was assayed in terms of units which were defined as follows: 1 PAU (prolan anti-unit) is "the smallest amount of anti-prolan required to annihilate the gonadotropic effect of 1 RU of prolan in a test rat." At least 10 PAU had to be used in an assay in a rat. PAU was found to circulate in effective amounts eight days after injection. If injected within, but not later than, twenty-four hours after the injection of PU extract, the ovulating effect of the hormone was prevented. Zondek & Sulman have also discussed various properties of PAU and contrasted these both with PU principle and anti-thyrotropic substance.

Okkels (297) has extended, by whole organ culture, previous findings that the thyroid glands of animals refractory to thyrotropic hormones are not themselves refractory but can be re-stimulated. Thyroids from rabbits, made resistant to an alkaline extract of beef hypophysis, were perfused in a Lindberg pump with fluid containing the extract to which the animal had become refractory. The thyroids responded as readily as control glands from previously untreated rabbits. Gordon *et al.* (298) secured evidence that the site of production of antihormones is the same as that of antibodies, i.e., the reticulo-endothelial system. They found that after splenectomy or reticulo-endothelium system blockage from injections with trypan blue, antihormones to pregnancy-urine gonadotropic extract appeared one to two weeks later than in control animals. Zondek & Sulman (299) were able by treatments with $N/10$ to $N/15$ HCl and $N/10$ to $N/15$ NaOH to destroy, selectively, either the prolan or the anti-prolan component of a prolan-antiprolan complex and so reactivate the other component. From their experiments they felt justified in making the assumption that antiprolan is not an antihormone in the strict sense, nor a ferment, but possibly a new kind of factor approaching very closely the immune bodies. They discuss the serological reactions secured by themselves and others and state, however,

that these do not favor the assumption that antiprolan is an antibody.

The work on antihormones for the year, thus, has not revealed the nature of the anti-substance. It is clearly established that refractoriness is not due to a loss of responsivity of the end-organ. Additional evidence indicates that treatment of a species with gonadotropic material prepared from that species does not lead to the development of refractoriness, i.e., antihormone formation. In treatments in which gonadotropic material from one species has been injected into a heterologous species, an inhibitory substance which usually is not species specific has been found almost invariably. Serological work has not clearly shown whether the substances inhibitory to the hypophysis are true antihormones or are immune bodies, perhaps with special or unique properties.

[The Editor is responsible for the spelling "oestrus," "oestrogens," etc., used in this review. The authors used the spelling recommended by the Advisory Council on Nomenclature of the Endocrine Principles, the American Medical Association, namely, "estrus," "estrogens," etc.]

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DEPARTMENTS OF BIOCHEMISTRY AND ANATOMY
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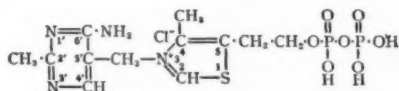
THE VITAMIN-B GROUP*

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VITAMIN B₁ (ANEURIN, THIAMIN)

Cocarboxylase and carbohydrate metabolism.—Last year the advances centred round the synthesis of vitamin B₁. This year an event of outstanding importance has been the discovery by Lohmann & Schuster (1) that cocarboxylase is a pyrophosphoric ester of vitamin B₁. The existence of this factor was first described by Auhagen (2), working in von Euler's laboratory. As Auhagen found that cocarboxylase was precipitable by lead acetate there seems to be little doubt that it is the combined form of vitamin precipitable by neutral lead acetate described by Kinnersley & Peters (3) in 1928. The compound has the formula C₁₂H₂₁O₇N₄P₂SCl; apart from the two phosphate groups it differs from vitamin B₁ in containing only one chlorine atom.

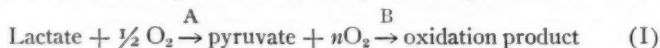


I. Cocarboxylase

As with inorganic pyrophosphate, one phosphate group is easily hydrolysed by normal acid, while the other is more resistant (1). Live yeast converts synthetic vitamin B₁ into cocarboxylase (4). Syntheses by enzymatic and chemical methods, namely by dried yeast plus adenosine triphosphate (5), by duodenal mucosa (6), and by means of phosphorus oxychloride (7) have been reported. The enzyme carboxylase in yeast, discovered by Neuberg in 1912, is concerned in the essential step in alcoholic fermentation by which pyruvic acid is converted to acetaldehyde and carbon dioxide; to obtain a completely active system cocarboxylase must be added as a supplement to specially washed yeast. Aldehydes inhibit the activity. Both manganese and magnesium salts improve it, whereas zinc and copper act as depressants. Neither vitamin B₁ nor its monophospho derivative can replace the pyrophosphoric ester (1).

* Received February 8, 1938.

The discovery of the chemical nature of cocarboxylase is important because it provides a definite function for vitamin B₁ in yeast metabolism and correlates this with its activity in animal tissues where it has been shown to be specifically concerned with the metabolism of pyruvic acid in brain (8). In a paper sent to press shortly before the appearance of the above work, Peters (9) had disposed of most of the evidence against the view that the vitamin was acting specifically upon the pyruvic acid, the system being considered to be:



Stage A is inhibited by fluoride; stage B takes place in the presence of vitamin B₁, but is inhibited by fluoride, iodoacetate, and dichlorodiethylsulfone. Peters added the point that the pyruvate-oxidase system in pigeon's brain was stabilised by both the vitamin and the substrate, which suggested that the vitamin was acting either as a coenzyme to this system, or as the prosthetic group of the enzyme. When Westenbrink & Polak (10) found a combination reaction prior to the start of the catatorulin effect, they were led to favour the prosthetic view.

While cocarboxylase has a definite function as such in yeast fermentation, its possible function in relation to pyruvic acid in animal metabolism is not yet clear, where lactic acid and not alcohol is the final product, and where vitamin B₁ itself is active *in vitro*. The claim by Lohmann & Schuster that cocarboxylase has the same action *in vitro* in the catatorulin test with pigeon's brain as vitamin B₁ itself has been disputed (11). It is stated that in catatorulin tests the former exerts not more than 10 per cent of the activity of the latter. The difference might not be fundamental and perhaps is due to the failure of the phospho compound to reach the active centre. Certainly it has been claimed that brain tissue synthesises cocarboxylase from vitamin B₁ in bicarbonate solution (1), and in catatorulin test conditions (11). Further, for some time cocarboxylase has been considered to be present in brain and to be decreased in avitaminosis (12), but it must be realised that it is still a hypothesis that vitamin B₁ acts in brain tissue as the pyrophosphate and it will be so until a preparation is obtained which interacts with the pyrophosphate and not with the uncombined vitamin. Only so can the activity of phosphatases, etc., be entirely excluded. In any case the system in brain tissue is different in other respects. There is no evidence of an anaërobic evolution of carbon dioxide with pyruvate (13, 11). Decarboxylation of α -ketobutyric acid by yeast carboxylase was shown to

occur by Neuberg & Kerb (14) in 1912. This requires cocarboxylase (11), but, nevertheless, this acid cannot be substituted for pyruvic acid in brain.

That cocarboxylase and not vitamin B₁ can be active in other systems was shown by Lipmann (13) with preparations of alkaline washed *Lactobacillus delbrückii* where it catalyses the aërobic decomposition of pyruvic acid into acetic acid and carbon dioxide. On the other hand Hills (15) finds that vitamin B₁ catalyses oxygen uptake with *Staphylococcus aureus* in the presence of pyruvate. Lohmann & Schuster suggest that pyrophospho vitamin B₁ as prosthetic group may combine with one protein in yeast and with another in systems such as brain.¹ As a stage in the activation of pyruvate, Lipmann (16) considers that the vitamin is hydrogenated, but there is no proof so far that such a change could be reversible and it is therefore still speculative. It may be noted that the pigeon brain-oxidase system is unstable and is inactivated by distilled water (17).

In the oxidation of pyruvic acid by pigeon-brain tissue, catalysed by vitamin B₁, 3.55 atoms of oxygen are taken up rather than the five theoretically required for complete combustion (18).² The fate of the incompletely oxidised portion is unknown. An examination of the possibility that the reaction proceeds by the dismutation scheme of Krebs (cf. 23), by some modification of the Toenissen-Brinkman scheme involving succinic acid, or by the α -ketoglutaric acid scheme of Weil-Malherbe, gives no experimental evidence for the belief that the main path of oxidation follows these directions (17). Weil-Malherbe (19) produces further evidence for succinic acid as an intermediary in brain metabolism, which is for the moment in direct conflict with the finding (17) that saturation of the succinoxidase system by succinic acid does not decrease the catatorulin-oxygen uptake with pyruvate. The possibility that acetaldehyde is acting as intermediary has received no experimental support (11, 20).

The above now proves that vitamin B₁ is directly concerned with intermediary carbohydrate metabolism, but it shows that in yeast its action is not directly concerned with an "oxidase" system. There is now the question whether it is generally concerned with sugar me-

¹ The evidence in this paper that vitamin B₁ and cocarboxylase have approximately the same value by the pigeon curative test cannot be taken as cogent in view of the recent criticism of this test for the pure vitamin.

² It must be noted that rat cerebrum is an exception in that pyruvic acid disappears with vitamin action without increase in oxygen uptake (24).

tabolism in the wider sense, i.e., movements of glycogen, etc. Much has been written upon this, but experimentally only the brain and kidney have as yet shown definite catatorulin effects *in vitro*. Other claims, even for a specific fall in tissue oxidation, have been definitely rejected (21). More recently again, Galvao & Pereira (22) consider that in pigeons there is no direct action upon muscle metabolism.³ It is particularly important to distinguish between possibilities and experimental proof in this field. The source of pyruvic acid in the blood in this avitaminous condition cannot be considered to be yet certain. Can the brain and the kidney account for all of it? Is it merely failure of experiment to demonstrate vitamin B₁ deficiency elsewhere, since there seems little doubt that vitamin B₁ must be of significance for cell metabolism in general? Of the carbohydrate disturbances established, in addition to pyruvic acid, we have the increases in lactic acid in blood (26), the change in the terminal stages in glucose tolerance (27), and possibly glycogen storage by the liver (28). Krusius & Simola (29), reinvestigating the rise of lactic acid in the blood of rats and guinea pigs in relation to avitaminosis generally, have concluded that diets deficient in vitamin A and vitamin C produce little or no change; deficiency of the total vitamin-B complex soon gives a marked rise; feeding autoclaved yeast (lacking B₁ and other thermolabile factors) gives a bigger rise which is not as great when autoclaved yeast is supplemented with vitamin B₁; feeding only vitamin B₁ gave an enormous increase. Hence it seems that the lactic acid changes are not associated only with vitamin B₁, but the value of these observations is somewhat reduced by the finding that the colorimetric method used for lactic acid estimation was not in agreement with other methods for avitaminous animals. The lactic acid increases may possibly be some reflection of the changes in pyruvic acid along the lines of equation I, but the changes in sugar are difficult to relate to the above.

One author definitely considers that changes in general carbohydrate metabolism are secondary to the dysfunction in the cells of the brain and elsewhere (30). There is now much support for the essentially "central" character of the symptoms. The matter is important clinically because of the question whether there is a direct connection with insulin action. Vorhaus, Williams & Waterman (31) have found that six out of eleven diabetics were improved as regards

³ Galvao (25) has proved also that the vitamin-B complex has no direct action on the total metabolism in pigeons.

carbohydrate utilisation by vitamin-B₁ administration. This is striking but the reviewers feel that these important effects must be secondary to a genuine nutritional deficiency; they know of no clear animal experiments relating vitamin B₁ with insulin action in this direction. Martin (32), for instance, investigated the action of insulin in pancreatectomised dogs in relation to nutrition and found that effects were produced by feeding with vitamins, but the results were inconsistent (cf. 33).

Clinical.—Closely connected with the above and conveniently considered here is the effect of dosing with vitamin B₁ upon alcoholic polyneuritis (34). Using the valuable data of Cowgill (35) as a basis it is held that with estimated inadequate vitamin B₁ for more than twenty-one days, alcohol addicts developed polyneuritis; with absolute deficiency, seven days might have been enough. Weiss & Wilkins (36) find that certain cardiovascular conditions respond readily to vitamin B₁. Selfridge (37) claims that lack of vitamin B₁ leads to demyelination of the VIIIth nerve in rats and chicks and that this is an important factor in inducing human deafness; clinical improvement by B₁ dosing is suggested. The above leaves no doubt of the practical importance of this vitamin in medicine.

Work upon the bisulphite-binding substances (B.B.S.) in blood is being continued. Following Platt & Lu (38), Wilson & Ghosh (39) state that B.B.S. is increased in epidemic dropsy (in India), anaemia, and splenomegaly, as well as diabetes. The substances concerned are not known. Wilkins, Taylor & Weiss (40) report estimations of B.B.S. upon 174 persons. Increases in several cases other than vitamin-B₁ deficiency were observed, indicating that the increase is not diagnostic of B₁ deficiency. Like Platt & Lu, they think that substances other than pyruvic acid, acetoacetic acid, and acetone contribute to the value. Shindo (41) isolated acetaldehyde as the 2,4-dinitrophenylhydrazone from beriberi blood. It is to be noted that in his excellent microchemical studies, Jong (42) found no direct relation between the B.B.S. increase in the blood of pigeons and the symptoms. Pi Suñer & Farran (43) isolated pyruvic acid as the 2,4-dinitrophenylhydrazone from the urine of compensated diabetics, and Johnson & Edwards (44) have found pyruvic acid in the blood and urine after severe exercise.

The problem of the metabolism of vitamin B₁ has been studied further in rats (45) and in humans (46). This is much like that of vitamin C in that the amount excreted is not nearly equal to the

amount ingested. In rats, faecal output is insignificant with intakes of less than 30 I.U. of vitamin B₁ daily. Upon a diet deficient in vitamin B₁, 0.1 to 0.4 I.U. was excreted in the urine daily which represents one-tenth of the amount disappearing from the body reserves. With higher intakes, up to 31 I.U., about one-third of the loss in body reserves appeared in the urine. Estimation of vitamin B₁ in the tissues by the bradycardia method confirmed previous work by others; the highest concentration stored (at intakes of 30 I.U. per day, a very large dose for a rat) is for the heart 2.7 I.U. per gm., liver 2.6 I.U. per gm., and muscle 0.6 I.U. per gm.; in terms of body weight 0.3 I.U. per gm. The stores of liver and muscle rapidly fell upon a B₁-free diet to levels of 0.1 I.U. per gm.

Chemistry.—There has been a steady consolidation of synthetic methods and exploration of the relation between structure and activity. Imai (47) has discussed the theoretical and experimental reasons which caused him to propose the present formula for the vitamin. Further details of the grounds which led Williams *et al.* (48, 49) to adopt this formula (I) have been described. The absorption spectra of mono- and dimethyl derivatives and non-alkylated derivatives of 6-oxy- and 6-aminopyrimidines are characterised by two absorption bands. Addition of acid markedly diminishes the prominence of the longer wave-length band whilst alkali tends to equalise the prominence of the two bands. The absorption of the aminosulphonic acid obtained by cleavage of the vitamin by sulphite shows the influence of the acidic sulphonic group and is affected in a similar manner as 6-aminopyrimidine on treatment with acid. Cleavage of the vitamin with liquid ammonia yields a base, C₆H₁₀N₄, whose ultraviolet spectrum is similar to that of the methylated 6-aminopyrimidine. The aminosulphonic acid, reduced with sodium in liquid ammonia, yields 2,5-dimethyl-6-aminopyrimidine and its structure is still further established from the fact that the action of sulphite on 2-methyl-5-ethoxymethyl-6-oxypyrimidine produces a 2-methyl-6-oxypyrimidine-5-methylsulphonic acid which is identical with the oxysulphonic acid derived from the vitamin. These investigations, coupled with synthesis, definitely establish the structure of the vitamin.

Two methods of synthesis of vitamin B₁ have been described in the past year. Cline, Williams & Finkelstein (50) have described details of their synthesis published in 1936 involving the reaction of 4-methyl-5-hydroxyethylthiazole with 2-methyl-5-bromomethyl-6-aminopyrimidine hydrobromide. Another and different synthesis of the

vitamin has been effected by Todd & Bergel (51) by condensation of 6-amino-5-thioformamidomethyl-2-methylpyrimidine with methyl α -chloro- γ -hydroxypropylketone. The synthetic vitamin has an m.p. of 230–232° agreeing with that of the vitamin isolated from yeast (52) but differing from the product obtained from rice polishings which has an m.p. of 249–250°. These differences in melting point of the synthetic and natural products are most satisfactorily explained on the grounds of dimorphism. The phenomenon is not confined to the hydrochlorides but is shown also by the picrolonates and the sulphates. The vitamin has been synthesised also by Andersag & Westphal (53) who presumably took out a patent for its preparation in January, 1936. The method adopted is similar to that of Williams and his colleagues. They have prepared also the isomer of vitamin B₁ in which the methyl group is in the 4' position instead of position 2'. This isomeric vitamin B₁ has a physiological activity less than that of vitamin B₁ itself, and it is of some interest to inquire what structural features are necessary to preserve the biological activity. From a study of several analogues of the vitamin Bergel & Todd (54) considered that the structural features essential for vitamin activity are the presence of a 6' amino group, a 5-hydroxyethyl group and a free 2-position in the thiazole nucleus. There is some evidence that change of the substituents in the 2' position may affect activity. These considerations are based on a study of the properties of the substances enumerated in Table I.

TABLE I
ANALOGUES OF VITAMIN B₁

Analogue	Position of Substituents*				Azo Test (57)	Thiochrome Test (58)	Activity
	2'	6'	2	5			
A (54)†	CH ₃	NH ₂	H	H	—	+	Slight to nil
B (54)	CH ₃	OH	H	CH ₂ CH ₂ OH	+	—	Slight to nil
C (54)	CH ₃	OH	H	H	—	—	Slight to nil
D (54)	CH ₃	NH ₂	CH ₃	CH ₂ CH ₂ OH	—	—	Slight to nil
E (54)	Cl	NH ₂	H	CH ₂ CH ₂ CH ₂ OH	Slight to nil
F (55)	CH ₃	OH	H	CH ₂ CH ₂ Cl	nil
G (56)	Cl	Cl	H	CH ₂ CH ₂ OH	nil

* Cf. formula I.

† The numbers in parentheses refer to citations in the bibliography.

Analogue F was prepared by Buchman & Williams (55) by treatment with concentrated hydrochloric acid. The reduction of vitamin B₁ with hydrosulphite, or with zinc and hydrochloric acid yields a yellow green intermediary compound which is considered to be a semiquinone in character (59).

Determination.—The present methods can be classified as: (a) vertebrate, based on rat growth, pigeon cure and protection (now discarded for pure vitamin) and rat bradycardia; (b) microbiological, using growth of the mould *Phycomyces Blakesleeanus* (66); (c) semibiological catatorulin test, using enzyme system *in vitro* for avitaminous pigeon brain; and (d) chemical, using the formaldehyde-azo reaction, the azo modification of Prebluda & McCollum (66a) and the thiochrome reaction (blue fluorescent oxidation product).

Of these, developments which promise well have taken place this year in the thiochrome method of Jansen and collaborators (60), using ferricyanide as oxidant (58). After oxidation in alkaline solution Jansen extracts with isobutyl alcohol and measures the fluorescence by a photoelectric method; Karrer & Kubli (61) use standards for comparison in place of the cell. The method has been applied to urine by Westenbrink & Goudsmit (62). After adsorption upon fuller's earth the product is eluted with alkali plus isobutyl alcohol, with and without ferricyanide. The control serves to estimate the residual fluorescence due to preformed products. Thiochrome methods have also been developed by Pyke (64) for the assay of food-stuffs. They are more sensitive than the formaldehyde-azo test which is nevertheless being further explored (65).

By use of the bradycardia method, Harris & Leong (67) record a value of 2.8 to 3.0×10^{-6} gm. for the International Unit. Scheunert & Schieblich (68), using rats, proved that the international standard clay remained stable for long periods. Williams (70) has pointed out the difficulties which have arisen from the use of the adsorbed solid clay as the international standard owing to the extra uncertainty of incomplete extraction in the animal gut. Sampson & Keresztesy (69) show that total extraction of the vitamin from acid clay can be made only with quinine salts.

Micro-organisms and vitamin B₁.—Schopfer and his colleagues (75, 76) have published some seventy papers during the last three years on the growth factors for the mould *Phycomyces Blakesleeanus*. The growth of this mould in a suitable medium containing asparagine is catalysed remarkably and quantitatively, within limits, by minute

amounts of vitamin B₁; 0.5 µg. in 10 cc. of media produces maximum growth. Views upon this have undergone rapid change. Originally thought to be vitamin B₂, the growth factor soon was considered to be vitamin B₁, but certain autoclaved products were also active. Recently, acting upon the suggestion of Knight (81), it has been found that the pyrimidine and thiazole fragments of the vitamin are equally efficient; this has been independently confirmed by Sinclair (77) and also by Bonner (85). The former has found that cocarboxylase is also active and has further confirmed the observation that degradation products produced by alkaline heating are effective. Hence the test is not specific and in reality is very similar to that described ten years ago by Reader for *Streptothrix corallinus* (78). In solutions where degradation products are unlikely to exist the method is valuable; in blood, for instance, the micro-organism provides the only present possibility for testing for the minute amount present (0.1 µg. per cc.). Meiklejohn (79) has adapted Schopfer's test to blood and found the apparent amount of vitamin B₁ to be some 30 µg. per 100 cc. in the pigeon, being reduced in avitaminosis. About 80 per cent is in the corpuscles (cf. 80).

Knight (81) has admirably cleared up the problem of the nutrition of *Staphylococcus aureus*. With vitamin B₁ (10^{-7} M) and nicotinic acid or amide (10^{-5} M) it can be grown in media of known chemical composition. Some pyrimidine and thiazole derivatives can be utilised (synthetic derivatives of A. R. Todd) but there is marked specificity, being confined only to those leading naturally to the synthesis of vitamin B₁. Schopfer has confirmed these results for his mould (82).

Kögl & Haagen-Smit (83) have demonstrated that vitamin B₁ has a marked stimulating action on the excised pea embryos affecting both the roots and shoots. In conjunction with biotin, a bios stimulating the growth of a species of top yeast, vitamin B₁ is still more effective. The biotin and vitamin-B₁ content of pea embryos compared favourably with the amounts needed to produce maximum growth experimentally. The relation of vitamin B₁ to biotin is at present obscure and its isolation from egg yolk by Kögl & Tönnis (84) has yielded no information in this respect. This substance is stated to contain nitrogen and sulphur but no phosphorus but possesses a marked blue fluorescence. Confirmation of the action of vitamin B₁ on pea embryos is found in the work of Bonner & Axtman (85). There is evidently wide variation in nutritional needs. The

work of Robbins *et al.* (86) shows that the thiazole part of the vitamin molecule alone is of value as a growth stimulant for tomato roots. Lwoff *et al.* (87), among other interesting points relating to growth factors of micro-organisms, find that *Polytoma candatum* and *Polytoma ocellatum* require thiazole only; *Glaucoma piriformis* requires vitamin B₁, while others can synthesise it from the components. Kögl & Fries (88) also have shown that some moulds require vitamin B₁, while others need also biotin.

Miscellaneous.—Comparisons have been made of the vitamin-B₁ content of white and brown bread (71, 72), flour, yeast, etc. (73). Changes in the amounts of vitamin A and vitamin D administered to rats do not influence their vitamin-B₁ requirements (74).

VITAMIN-B₂ COMPLEX

Nomenclature.—Within recent years evidence has been presented for a number of factors required by different species for normal development. The tendency to apply different terms to the same factor with its attendant confusion warrants some mention of the nomenclature used in this group of accessory factors. The name "vitamin B₂" or "vitamin G" was employed to denote the factor curative of dermatitis in the rat. The isolation from vitamin-B₂ extracts of riboflavin (lactoflavin), a substance essentially growth-promoting in its action, and the definition of vitamin B₆ (89) and factor Y (90) have led to the adoption by some of the term vitamin B₂ or vitamin G for flavin. Still greater confusion has arisen from the extensive investigations into chick nutrition.

In addition to vitamin B₁ and flavin the chick requires a factor called the "filtrate factor" or "factor 2" by Lepkovsky *et al.* (91) but "vitamin B₂" or the "antipellagra factor" by Elvehjem (92). These terms are applied to impure concentrates. Previous to 1936 it was generally considered that the rat required vitamin B₁, flavin, and vitamin B₆ whose nature might be composite. Lepkovsky, Jukes & Krause (91) found that the rat needed the filtrate factor of the chick together with another factor separable from it by adsorption on fuller's earth which they later called "factor 1." The position of vitamin B₄ is again obscure. It has been stated that Reader (93) really worked upon two vitamin-B₄ factors, one growth-promoting to young rats and precipitable by mercury salts, the other, adsorbed on acid charcoal, curative, when administered with vitamin B₁, of certain symptoms of the adult rat (94). The former factor was probably an early hint of flavin, while the latter work has not been repeated.

The symptoms of some of these rats resembled pink disease in man very closely, much more so than rats suffering from vitamin-B₆ deficiency which György subsequently termed rat acrodynia. Lately Elvehjem *et al.* (95) have applied the term vitamin B₄ to a factor present in acid charcoal extracts prepared according to the procedure of Kinnersley *et al.* (147), which is curative of a form of chick paralysis. Elvehjem's vitamin B₄ therefore is not necessarily identical with Reader's vitamin B₄. In the case of the pigeon the position has remained stable since 1930 when Carter, Kinnersley & Peters (96) added vitamin B₅ to the vitamin B₃ of Williams & Waterman (97), except that Carter & O'Brien (98) now consider that, in addition, flavin and casein are needed for complete weight restoration. It appears from this year's work that the concentrates of the filtrate factor may be curative not only of blacktongue (99, 100) in the dog but also of pellagra in humans (101). The problem now before workers in this field is to isolate the factors, at present known only by their effects, and to determine whether in some cases apparently different factors may be identical and so to indicate by exclusion whether there are still others unknown.

Vitamins B₄, B₆, and the filtrate factor.—Beyond the introductory mention there is no fresh information on vitamin B₄. The simple picture, that vitamin B₂ consisted of riboflavin and the rat-antidermatitis factor, vitamin B₆, has of course broken down. This was suspected for some time in some laboratories engaged in this work but the clearest initial publication was that of Lepkovsky, Jukes & Krause (91) who found that the third factor required by the rat could be resolved into two components (required for complete growth) by a fuller's earth treatment of an extract of rice bran which adsorbed the rat antidermatitis factor (their "factor 1" or "vitamin B₆," which did not cure chick dermatitis).

The other factor present in the filtrate-factor concentrate was needed to supplement rat nutrition and cured chick dermatitis (chick pellagra). In confirmation of this work are the results of Halliday & Evans (102, 103). With extensive alcoholic extraction of the casein of the diet to remove traces of flavin and substitution of sucrose for corn starch these workers were able to produce dermatitis consistently in the rat. Brewer's yeast, liver, whole wheat, and extracts of these and of rice bran were potent in the antidermatitis factor, vitamin B₆. A concentrate of the latter was prepared by adsorption on fuller's earth and elution with baryta solution, a procedure similar to that of

Lepkovsky *et al.* The supplementary action of the concentrates of the filtrate factor was also observed. In agreement with the work of Lepkovsky *et al.* (91), Edgar & Macrae (104, 105) have produced very clear evidence for the presence in yeast extracts of two heat-stable factors essential for rat growth. One factor, the yeast-extract filtrate factor (? filtrate factor), is present in the filtrate after exhaustive treatment with fuller's earth of an acid-autoclaved yeast extract; the other, resembling vitamin B₆, is adsorbed on fuller's earth and can be eluted with baryta as shown by Lepkovsky *et al.*, and by Halliday & Evans. According to the latter it is fairly stable towards alkali at the temperature of a boiling water-bath.

The bipartite nature of the third rat factor permits suitable interpretation of the results of Euler, Malmberg, Schlenk & Gleim (106) who have investigated the properties of this factor. Their active component was precipitated by mercury and silver salts, a property not characteristic of vitamin B₆ (89). In view of this difference they have designated the factor B_v. Booher (107) also considers that the concentrates she has obtained from whey and rice polishings possess the properties of a mixture of the different factors required by the rat, i.e., filtrate factor, vitamin B₆, and factor W.

Sjollema (108) reports that isoleucine cures dermatitis. Muus *et al.* (109) find that there is a 24 per cent decrease in liver metabolism whilst that of the diaphragm is normal in vitamin-B₆-deficient rats.

Filtrate factor (for chicks).—According to Jukes (110) this factor is extracted easily from feed by hot or cold acidified water. The best source is yeast and rice bran but it is present also in egg yolk and in small amounts in cereals. It is stable to benzylation, hardly precipitable by phosphotungstic acid and is soluble in 95 per cent ethanol.

Filtrate factor (for rats).—Edgar & Macrae (104, 105) find that the factor in their extracts is stable to light, not precipitated by basic lead acetate, mercuric sulphate or basic precipitants but is precipitated by barium hydroxide in 90 per cent ethanol which suggests that it is acidic. The properties resemble those of the preceding factor for chicks.

Nicotinic acid, nicotinamide, and factor W.—The biological importance of the pyridine compounds, recently recognised by the demonstration of Warburg & Euler that nicotinamide is the active group in certain coenzymes, has led to the exploration of other fields for the utilisation of these substances in nature (cf. 81). The isola-

tion of nicotinic acid from potent antineuritic concentrates by Funk some twenty years ago suggested that it might be related to vitamin B₁ but it was inactive as the antineuritic substance. More recently Elvehjem, Madden, Strong & Woolley (111) have claimed that both nicotinic acid and its amide are curative of blacktongue in dogs. There is some evidence that the amide is active in promoting the growth of rats (112, 113) and pigeons (114) on diets deficient in some factor of the vitamin-B complex. Euler & Malmberg (115) also consider that, in contrast to cozymase, nicotinamide stimulates the growth of rats. On the other hand, it appears from the results of Edgar & Macrae (116) that these pyridine compounds cannot replace their yeast-filtrate factor or yeast-eluate factor although these workers have isolated nicotinamide from concentrates of the yeast-eluate factor. Moreover, Dann (117) has confirmed the curative action of nicotinamide on canine blacktongue but finds that it has no effect upon the dermatitis of chicks or of rats. This is of interest since Elvehjem and his colleagues (112, 113) claim to have isolated from concentrates of the antipellagra factor (chick-filtrate factor ?) a substance agreeing in melting point and analysis with nicotinamide. It is also stated that cure of monkeys showing pellagra-like symptoms is possible with nicotinamide.

In addition to vitamins B₁, B₄, B₆, flavin and the filtrate factor, Elvehjem, Koehn & Oleson (118) think that the rat requires still another factor which has been termed "factor W." This factor, precipitable from liver extract by mixtures of alcohol and ether, is extremely active in promoting growth in rats but has no effect upon the dermatitis of chicks. Although not adsorbed upon fuller's earth there is some doubt whether it is distinct from vitamin B₆ (119). In a more recent paper Elvehjem & Frost (113) report evidence suggesting a relation of this factor to the pyridine nucleotides. The complementary action of flavin and factor W to one another and the chemical behaviour of the latter towards mercury and barium salts indicated a similarity to the coenzymes. A definite growth response was claimed on addition of nicotinamide and adenylic acid to the diet, which was enhanced by the simultaneous administration of these two substances. It must be realised, however, that at present results on "factor W" are difficult to reconcile with other findings owing to the non-synthetic nature of the diet. Vitamins B₄ and B₆ were provided in the form of white corn composing 12 per cent of the diet which, it is stated, also contain small yet adequate amounts of the filtrate factor.

Nutrition of monkeys and pigs.—Further attempts to effect a more definite link between similar symptoms in animals have led to a study of the nutritional requirements of other animals than the rat and the dog. The feeding of monkeys (*Macacus rhesus*) on a Goldberger P-P diet, rich in vitamin B₆ and containing traces of flavin, produced profuse diarrhoea often with vomiting, denudation of the fur, dryness and roughness of the skin, and scaliness of the hands and feet, a response similar to that seen in pellagrins (120). Although not definite, the administration of flavin did not appear to influence the condition. Yeast, liver powder, and wheat produced rapid cures. A similar result has been obtained by Birch, Chick & Martin (121) by feeding pigs diets of the Goldberger blacktongue type and modelled on diets consumed by pellagrins. Yeast and autoclaved yeast extracts were good curative agents. Birch, Chick & Martin (121) were able to rear young rats satisfactorily on their diets but Harris (120) observed a submaximal growth unaccompanied by active lesions which could be corrected by the administration of liver.

Lactoflavin.—The relation of lactoflavin to oxidase systems is discussed elsewhere. Interest has shifted from special work upon this known factor to the unknown entities, but Kuhn & Ströbele (122) have produced chemically the green and red intermediates in the oxidation-reduction system. Kenner (123) draws attention to the true nature of this change in which he thinks there is removal not of two hydrogen atoms but of a proton and hydrogen atom associated with an electron. Kuhn & Boulanger (124) have shown that some alloxazines are toxic in small doses. Emmerie (125) describes his method of estimation. It is noticeable that the amounts of lactoflavin necessary to produce maximal effects in rat diets are on the increase. Edgar & Macrae (126), for instance, use up to 30 to 50 µg. per day. Though not pellagra-preventative (cf. 127) or blacktongue-curative in doses of 10 mg. Sebrell, Hunt & Onstott (128) consider flavin necessary for dog nutrition. However, the dermatitis of turkeys (resembling that of the chick in symptoms) is cured apparently by 2 mg. of this factor per 100 gm. of diet, a curious instance of specificity (129).

The growth response of chicks has been shown by Jukes (130) to bear a linear relation to the amount of lactoflavin administered and he has utilised this bird to assay the factor in different foodstuffs; prolonged deficiency induced changes in stance and a "crow-like" head. Distribution of flavin in foodstuffs has also been investigated

by Levine & Remington using rats (131). Further confirmation of the relation of flavin to alopecia and cataract is found in some careful experiments of Day, Darby & Langston (132). Rats on synthetic diets, carefully freed from flavin and supplemented with vitamins B₁ and B₆, developed alopecia and cataract in four to twelve weeks, whereas rats receiving crystalline lactoflavin showed no lenticular changes. Some interesting experiments of Euler & Malmberg (133) indicate that extracts of top yeast may contain some factor capable of replacing flavin in the diet of rats.

In continuation of the work reported last year⁴ Hubner, Laszt & Verzár (134) find that in rats and cats after extirpation of the adrenals there is a strong decrease in the amount of flavin in the liver combined as "yellow enzyme." By a process of exclusion of vitamin B₁, "vitamin B₄," vitamin B₆, and lactoflavin, they conclude that lactoflavin phosphate must be the factor in yeast extracts permitting growth with adrenalectomised animals; they even consider now that it can be substituted for vitamin B₆ as a curative agent (135, 136).

Pellagra.—Birch, Harris & György (137) considered that the factors curative of blacktongue and pellagra might be identical and distinct from vitamin B₆. Flavin has been excluded as the curative agent by Dann (127) and by Sebrell, Hunt & Onstott (128). Excellent therapeutic results have been obtained with liver preparations in the treatment of pellagra and canine blacktongue. Concentrates of the antidermatitis factor of the chick from liver and rice bran have been shown to be curative of pellagra (101) and blacktongue (99, 100). Although consideration of this fact, and the similarity of the symptoms with those of the dog, pig, and monkey, might lead one to presume that only one factor is involved, the more recent studies in pellagra suggest that the problem is more complex. Aykroyd & Krishnan (138) found that angular stomatitis in Indians is rapidly cured by autoclaved yeast. Some preliminary therapeutic trials on pellagrins in Egypt by Ellinger, Hassan & Taha (139, 140) suggest that improvements may be effected, not only by the concentrated yeast-eluate factors but also by the yeast-filtrate factor prepared by the method of Edgar & Macrae. Ruffin & Smith (141) have also concluded that the P-P factor of Goldberger might be composed of two or more substances; they found that while an aqueous extract of liver produced complete recovery, parenteral extracts of liver alleviated only the tongue symptoms. Sydenstricker & Thomas (142) advanced

⁴ *Ann. Rev. Biochem.*, 6, 308 (1937).

the hypothesis that two factors are involved, an intrinsic one contained in gastric juices and an extrinsic one, possibly contained in the vitamin-B₂ complex.

In clinical studies, Beckh, Ellinger & Spies (143) found a high correlation between coproporphyrinuria and pellagra, the condition disappearing upon cure. A more intensive research on this problem should yield interesting metabolic results.

Egg white and factor H.—The term vitamin H appears to denote different factors. According to György it is the component in foods capable of counteracting the deleterious effect of excessive amounts of egg white in a diet, and since it is rendered water-soluble after enzymatic hydrolysis of the foodstuffs, it does not really fall in the class of the vitamin-B complex. György has recently produced evidence to show that it is an amino acid (144). Booher (107) uses the term to denote the residuum necessary, in addition to vitamin B₁ and flavin, to complete the growth-promoting activity of the vitamin-B complex and to prevent erythrodermic dermatosis in the rat, a definition which fits the old description of vitamin B₆.

Anaemia and the vitamin-B complex.—Interest in the relation of certain types of anaemia to factors of the vitamin-B complex has been revived. Hogan, Richardson & Johnson (145) found that pigeons on a synthetic diet developed a form of anaemia which is cured by yeast but not cured by vitamin B₁, flavin, or the antidermatitis factor. Also Clutterbuck, Evans & Wills (146) consider that some factor, probably related to the vitamin-B₂ complex, is curative of anaemia produced experimentally in the Rhesus monkey by dietary means.

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VITAMIN C (ASCORBIC ACID, CEVITAMIC ACID)*

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The year has been one of consolidation and critical appraisal of previous claims.

Chemistry.—Papers upon the organic chemistry of this vitamin are naturally few. Haworth, Hirst & Jones (1) have taken exceptional care to prove that *D*-gluco-ascorbic acid really has the structure assigned. The *L*-form is slightly active. Several physico-chemical points are now clearer. A slight increase in the earlier pK values of Birch & Harris and Karrer *et al.* by Ball (2) gives pK 4.21 instead of 4.17. A careful study of oxidation-reduction potentials has been made by Borsook, Davenport, Jeffreys & Warner (3). They recognise at least three stages *in vitro* corresponding to the previously recognised stages of degradation (4) but they emphasise that the correctness of their facts does not depend upon this interpretation. The first stage, A, can be studied at a pH lower than 5.0 (at pH 5.0 $E_o' = +0.127$) and corresponds, presumably, to the reversible transformation ascorbic acid \rightleftharpoons dehydroascorbic acid. The oxidised (and only the oxidised) dehydro form passes very readily through two other stages, the transformation occurring with increasing rapidity as the pH becomes greater than 6.0. The products of both changes are more strongly reducing than A. The changes correspond to the transformation from dehydroascorbic acid to diketogulonic acid (B) ($E_o' = +0.015$ at pH 5.5), and to the further degradation (C) to decomposition products previously characterised as *L*-threonic acid and oxalic acid. Stage A was measured electrometrically at pH < 5, equilibrium being slowly attained, and stages B and C with oxidation-reduction indicators. Values for A have been confirmed by Ball (2) using as carrier traces of dye which induces a more rapid attainment of equilibrium. He criticises the figures for B and C upon the ground that no true reversibility is shown and that velocities do not necessarily indicate the true values. Only stage A is normally important biologically¹ and the importance of keeping ascorbic acid reduced

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¹ Cf. *Ann. Rev. Biochem.*, 6, 354 (1937)

in the tissues is clearly shown by the extreme lability of the dehydro form at body pH. The only case in which dehydroascorbic acid has been proved to exist as such is in the apple (5), though many living systems rapidly reduce this form. Stage B can, at present, be reversed only by chemical means (4), and there is no evidence that B and C have biological significance. In the paper by Borsook *et al.* (as elsewhere) is to be found clear evidence that glutathione (originally shown by Szent-Györgyi to interact) is one of the main constituents of blood responsible for the stability of ascorbic acid. No dehydroascorbic acid exists in blood; its alleged presence is an artifact due to oxidation *in vitro* by the precipitants at the moment of precipitation of blood proteins in the presence of blood corpuscles (3, 6, 7). Reference may be made to the work of Fischer (8) on the behaviour of ascorbic acid *in vitro* with haemoglobin compounds and cyanide in which an adsorption compound with oxyhaemoglobin is obtained (cf. 9).

Kon (10) finds that visible light destroys the vitamin C in milk, an observation of practical importance. A clue to the mechanism of this has been given by Hopkins (11). In the presence of light *in vitro* the fluorescent lactoflavin rapidly causes destruction of the vitamin, there being a simultaneous degradation of lactoflavin to lumichrome, i.e., an interaction of two vitamins. Another action *in vitro* of ascorbic acid has received attention, namely the deaminating action upon amino acids (12, 13). There is no evidence that it is of biological significance.

Methods of estimation.—Papers upon this subject are very numerous and various methods have been tried (3, 14, 15, 16, 17) though there is not much change in the position since last year's review. The most practical method for urine still seems to be acidification to pH 3 and titration as soon as practicable, though the presence of other reducing substances may interfere (18, 19). In plant juices the total reducing power to 2,6-dichlorophenolindophenol probably corresponds to the ascorbic acid present, but in animal-tissue extracts, particularly those of liver tissue, it may be as much as 20 per cent too high.

Ascorbic acid oxidase.—There is still much interest in the capacity of oxidising ascorbic acid possessed by plant extracts, one of the first facts to be described by Szent-Györgyi. The properties of a series of such oxidases have been reported by Johnson & Zilva (20). They conclude that there are slight differences in matters of detail in

respect to their activities, pH range, and behaviour to cyanide. In the cabbage, cauliflower, cucumber, and marrow the enzymes oxidise *l*-ascorbic acid directly, but in the apple and potato the phenolases present oxidise it in the presence of catechol or the juice. Tauber (21) has described the oxidation of ascorbic acid by plant peroxide-peroxidase systems when there is present some substance capable of acting as a carrier by forming a quinone, but it must be remembered that any oxidising system which forms an oxidant of the correct potential should be capable of oxidising unprotected ascorbic acid *in vitro*.

In another paper (22), following up the previous finding of Zilva that the enzyme is not specific for *l*-ascorbic acid, Johnson and Zilva find that the members of a series in which the ring is to the right of the carbon chain (namely, *l*-ascorbic acid, *d*-arabo-ascorbic acid, *l*-gluco-ascorbic acid, and *l*-galacto-ascorbic acid) are directly oxidised by cucumber oxidase at much higher rates than are their enantiomorphs (for formulae see 22). In the slowly oxidisable group oxidation is faster for those with six than with seven carbon atoms. The facts suggest that the asymmetric carbon atoms outside the ring also condition antiscorbutic activity but not the direct enzyme oxidation of *l*-ascorbic acid and its six-carbon analogues.

In contrast to the specificity implicit in the above, Stotz, Harrer & King (23) regard the enzyme as an association resembling Bertrand's artificial laccase. Minute traces of copper previously have been reported² to catalyse actively ascorbic acid oxidation, and Stotz *et al.* state that they can imitate the enzymatic oxidation with copper + gelatin or copper + albumin. The argument is supported with evidence that copper inhibitors reduce the activity of artificial and natural enzymes in a similar way (a discrepancy for cabbage juice is explained), that there is a general resemblance of activity-pH curves, that heating to 100° C. for five minutes destroys the activity, and that similar inactivation is effected by treatment with acid at pH 2.0. They report only 60 to 70 per cent inactivation as a result of two hours treatment with acid, while Tauber *et al.* (24) report total inactivation of the natural enzyme in fifteen minutes. In the case of inactivation of the artificial enzyme, the copper is presumably removed from the solution by precipitation of the protein or converted to some inactive association compound. The case made out by these

² Cf. *Ann. Rev. Biochem.*, 5, 370 (1936).

authors requires consideration but it would have been improved by publication of copper estimations on the natural enzyme; it does not yet seem to account for the apparent specificity.

Mode of action.—There is a strong feeling that vitamin C acts by virtue of its reducing properties. Furthermore there is evidence that its absorption by tissues of the rat and guinea pig is specific as compared with inactive compounds of the series (25). However, there has been no progress in demonstrating an effect of the vitamin upon ascorbic-avitaminous tissues. The claims originally made by Harrison (26) and by Quastel & Wheatley (27) for an action, *in vitro*, of ascorbic acid upon scorbutic and normal liver tissues have turned out, upon closer examination, to be fallacious. Stotz, Harrer, Schultze & King (28) conclude that the rates of oxygen consumption and carbon dioxide evolution of guinea-pig liver (but not kidney) tissues increase with onset of scurvy and that there is no increased oxygen consumption, as the result of adding ascorbic acid, over and above that due to oxidation of the ascorbic acid itself. There is, therefore, nothing described yet with these tissues analogous to the catatorulin effect of vitamin B₁, but there is the older observation (27) that addition of ascorbic acid to rat-liver slices partially removes the inhibition of acetoacetate production caused by iodoacetate. Scheunert & Schieblich (29) have found that the livers of rats upon diets free of vitamins A, B, C, and D still contain sufficient ascorbic acid to cure guinea pigs; hence the synthesis of ascorbic acid is independent of these factors.

Ascorbic acid and toxins.—Related to the above is the question of the role of ascorbic acid in toxic conditions. There is indication that the ascorbic acid content of the adrenals of guinea pigs is diminished in diphtheria intoxication (30). Papers still appear indicating that the general level of nutrition of guinea pigs as influenced by the quantity of the food intake and by ascorbic acid is significant. For instance, Sigal & King (31), using the glucose-tolerance test, showed that sub-lethal injections of diphtheria toxin caused a greater carbohydrate disturbance in guinea pigs receiving just enough ascorbic acid to prevent scurvy than in those receiving a more generous supply. But the ascorbic acid dosing of animals not subject to scurvy, e.g., mice (32) and rabbits (33), has been shown to be without effect on the course of intoxications or infections.

In a recent paper Zilva (34) discusses critically the literature upon the alleged antitoxic action of ascorbic acid in diphtheria. He

points out that a diminution of ascorbic acid in the adrenals or other organs of guinea pigs suffering from diphtheria is probably secondary and that even if destruction of the toxin by the acid occurs *in vitro* it is not of significance *in vivo* since many substances carry out such inactivation *in vitro*. Using guinea pigs he attacks again the problem of whether or not ascorbic acid can modify the action of diphtheria toxin *in vivo*. Known amounts of diphtheria toxin were injected into animals (a) depleted, (b) saturated, or (c) injected with ascorbic acid. In these three conditions the plasma "apparent" ascorbic acid was (a) practically nil, (b) 0.5 mg. per 100 cc., or (c) from 32 to 3.5 mg. per 100 cc. for periods of from one to four hours after injection. The tables show no differences among the groups. Animals with or without ascorbic acid reacted identically to one minimum lethal dose of toxin.

In addition, attempts to prove an undoubted action of ascorbic acid on toxins *in vitro* have been seriously criticised by Sigal & King (35) who state that when ascorbic acid is carefully neutralised to pH 6.4 to 7.4 before being mixed with diphtheria toxin there is no inactivation. The published data of other workers do not appear to exclude the possibility that the alleged inactivation was caused by the non-specific acid or reducing properties of ascorbic acid. No experiments using the closely related but non-antiscorbutic substances have been reported.

Such evidence appears to the reviewers to be convincing, though of course it does not exclude the possibilities that ascorbic acid may be catabolised to a greater extent by diseased animals or that it may have an inhibitory effect upon the growth of microorganisms *in vivo*. But this is speculative. It is quite clear to an impartial observer that no case has yet been made out for ascorbic acid as an antitoxic agent *per se*, that is apart from its influence as an antiscorbutic agent.

Metabolism and the significance of saturation.—Much attention still continues to be devoted to the relation between the amount of ascorbic acid in the plasma and in the urine. Most of the work is of a purely clinical nature, but the question is also of scientific interest. The facts established in previous years are briefly as follows: The ingestion of constant doses of ascorbic acid by man or animal gives rise after a variable time to an increased excretion in the urine³ which

³ Proved originally by the urine's curative action on guinea pigs. Recently, ascorbic acid has been isolated from urine as the 2,4-dinitrohydrazone (36, 37).

tends to reach an approximately constant value (38, 39, 40); with large doses this constant amount may be some 300 mg. in twenty-four hours, but it is always much less than the amount ingested. The fate of the disappearing ascorbic acid is unknown, but Wright *et al.* (41) and Bernstein (42) have reported the excretion of ascorbic acid in sweat. This is not only important for miners, who often suffer from sub-scurvy, but it might also help to explain the greater disappearance of ascorbic acid in patients with diseases causing increased sweating. There is a low urinary excretion of ascorbic acid in scurvy, and the excretion is raised after the ingestion of ascorbic acid. There is a tendency for the excretion to rise more rapidly after large oral doses in conditions in which much ascorbic acid has previously been given, though the excretion falls upon the cessation of administration. Thus there is evidence of "saturation." In guinea pigs, even when two to three times the necessary daily protective dose is given the selective tissues contain only traces of ascorbic acid (43). To get maximum tissue saturation ten times the protective dose is necessary. Yet both groups of guinea pigs become scorbutic at approximately the same time after having been placed on a scorbutic diet; hence the tissue vitamin C hardly behaves as a store. In patients all signs of scurvy disappear during ascorbic acid dosing some time before there is any evidence of saturation (44). Normal individuals do not respond to test doses of ascorbic acid with increased excretion at equal rates.

The present problems are: (a) whether the normal urinary excretion is a guide to the general state of the organism; (b) whether the measure of saturation is significant; (c) whether non-scorbutic degrees of "unsaturation" are dangerous to health. This last point is to be distinguished from such questions as to whether a scorbutic animal gets infected more easily than a vitamin A or D deficient animal. [See Perla & Marmorston (45) for a review of these questions.] In regard to (a) Abbasy, Hill & Harris (46) have stated from massed experiments upon hospital patients that rheumatic, rheumatoid, arthritic, and tubercular patients show upon the whole low excretion values even when being dosed with liberal supplies of orange juice, the amounts of which were controlled in one paper. Finkle (47) found generally low excretory values in hospital patients.

As for (b) there are two definitions of saturation. Some consider that the rise in excretion following one or, at the most, three test doses of large amounts of ascorbic acid is sufficient to give evidence

of the degree of saturation (47). Others (39, 44) believe with justification that such values may be unreliable and that dosing until constant excretion is reached provides the only true measure of the condition. Recently Wright *et al.* (48) state that saturation is best measured by vascular injection of ascorbic acid. Abbasy *et al.* (46) find that their patients excrete relatively less than do normal persons in response to three test doses. In some cases the results are striking, and some of the facts are supported by other evidence. Finkle (47), using an injection technique, found low saturation as well as low excretion in lupus erythematosus and also in rheumatoid arthritis. These results led them to the general view that there is a greater apparent utilisation of ascorbic acid in these cases, but it must be noted, as Parsons (49) has pointed out, that the findings of Abbasy *et al.* do not apply specifically to rheumatism. He and his colleagues found that rheumatic patients tended to excrete more than "infected" controls.

(c) The implicit interpretation that there is a direct relation between these states of apparent unsaturation and the disease does not seem clear, and it cannot yet be accepted at its face value. The results are not really concordant with careful observations by Sendroy & Schultz (50, 51) who do not think that dosing with ascorbic acid improves the rheumatic condition. This view is supported by Parsons (49) and by Baumann & Rappolt (14) for other infections. There is no evidence from animal experimentation that saturation, as such, matters.⁴ In fact it has yet to be demonstrated that any person requires an excess of vitamin C beyond a wise nutritional margin of at least twice the indispensable minimum to protect against scurvy and latent scurvy. The latter has been redefined in careful work by Göthlin (52) using capillary-fragility tests as 23 to 29 mg. per 60 kg. for the normal person. For pregnant and nursing women the amount would be increased to three times. For the latter 50 mg. per day is given by Baumann & Rappolt (14) and 80 to 100 mg. per day by Widenbaner & Kühner (53). "Gastric" cases need more. It is to be noted that metabolic conditions certainly influence excretion. Less is stated to be excreted in urine under alkaline than under acid condi-

⁴ As a qualification to this Perla (45) reports one unpublished experiment indicating that extra vitamin C protected guinea pigs against trypanosome infection. If the basic diet was adequate in vitamin C this experiment would certainly support the idea that partial saturation was helpful.

tions; conversely, that the liver retains more. Drugs as well as anaesthetics also influence excretion (54, 55, 56).

The number of papers upon vitamin C and related subjects is very large this year and it has been impossible in this short review to do adequate justice to much of interest. Reference may be made for instance to Glick (57) for microtechnical achievements and determinations in the developing shoot, to Zilva & Morris (58) for proof that the vitamin-C content of canned vegetables and fruit can be improved by adding vitamin C, to Perla & Marmorston (45) for a review of vitamin C in pathology, and to Giroud *et al.* (62) for the distribution of ascorbic acid in tissues of the ox and horse.

Vitamin P.—Considered to influence permeability especially, and identified by Szent-Györgyi (64) and collaborators as citrin and hesperidin, this vitamin has not yet established itself beyond criticism. Starting from the view that the factor was active clinically in man (purpura conditions) Szent-Györgyi and associates thought at first that it prolonged the life of guinea pigs and lowered the intensity of the pathological lesions. This could not be confirmed by Zilva (59), who stated that he could reproduce the animal symptoms by traces of ascorbic acid below the curative dose. Later, Szent-Györgyi and colleagues took the view that the factor required small traces of ascorbic acid for its activity (60). Moll's experiments (61) have confirmed Zilva in finding no curative effects with hesperidin, etc., nor interactions with small doses of vitamin C in guinea pigs. The efficacy of vitamin P in guinea pigs is, therefore, unconfirmed. Whether there are any clinical effects is undecided; it is certainly curious that Warburg & Elmby (63) state they have cured scorbutic patients (and raised serum ascorbic acid) by the use of lemon juice in some cases not cured by ascorbic acid. Such suggestions make it unwise to pronounce sweepingly against the existence of a capillary factor for man.

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THE FAT-SOLUBLE VITAMINS*

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GENERAL

Molecular distillation.—A group of workers in the Research Laboratories of the Eastman Kodak Company have made an important contribution to the technique for isolating substances of the type of fat-soluble vitamins. A new form of molecular still has been devised which has many advantages over the type constructed by Burch (1) which had already demonstrated its value in concentrating vitamin A. Theoretical considerations underlying the new process are outlined by Embree (2), the general plan and operation of the apparatus are described by Hickman (3), and certain relevant practical details by Baxter, Gray & Tischer (4). In another paper Hickman (5) records how the apparatus was used to study the state of vitamins A and D in fish-liver oils and how they were successfully distilled from the oils themselves. Cod- and halibut-liver oils contain vitamin A both in the free state and esterified, the two forms being readily separable by distillation. A very ingenious feature of the process is the use of pilot dyes in following the distillation of an unknown substance. Work on cod- and tuna-liver oils suggests that most of the vitamin D is present in the esterified form. The process is so successful that a pilot plant is already producing enough pure vitamin A to supply one-tenth of the demand in the U.S.A. (6).

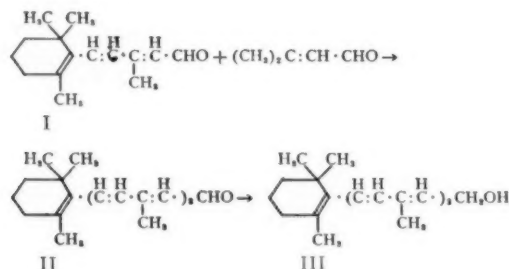
Fat-soluble vitamins in margarine.—A series of papers in the Danish journal *Ugeskrift for Læger* brings home to one the difficulty and complexity of the task of legislating to improve the diet of the working people. Memory of the outbreak of xerophthalmia during the World War years when the poorer people in Denmark ate little butter, revived by recent revelations that a considerable proportion of the working population is still living on diets inadequate in respect to vitamin A, led the Government, on the recommendation of Professor Møllgaard, to introduce a law that all margarine must be "vitaminised." Recent examination of such fortified preparations shows, however, that the vitamin A content is only a little higher than that of

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winter butter, while the vitamin D content is only one-fifth of the value of 0.5 I.U. given for ordinary winter butters. Recommendations from experts that a higher level of reinforcement is necessary if the measure is to be of any practical value have led to a vigorous protest from the farming interests that the sales of butter will be affected unless they, too, are permitted to fortify. These thirteen papers present almost every side of a very complex subject (7).

VITAMIN A

Chemistry.—Two outstanding events mark the year under review, a synthesis of vitamin A and its isolation in a crystalline form from a natural source. The former achievement is announced from Kuhn's laboratory at Heidelberg (8). β -ionone was condensed with bromoacetic ester to give ethyl- β -ionylideneacetate, which was caused to react with the product obtained by treating *o*-toluidine with methylmagnesium iodide. The resulting β -ionylideneacet-*o*-toluidide was converted, via the imidochloride, to β -ionylideneacetaldehyde (I). Then the following condensation was carried out, giving rise to the aldehyde of vitamin A (II), which was subsequently reduced by aluminium isopropylate to the alcohol itself (III). The yield was not good but identity with natural vitamin A of material isolated after subjecting the crude product to chromatographic adsorption is rendered highly probable by the results of feeding experiments with rats.



Synthesis of vitamin A

The story will be complete if Kuhn now applies to his impure synthetic product a process such as that by which Holmes & Corbet have successfully prepared crystalline vitamin A from the unsaponifiable fraction of the liver oil of *Stereolepis ishinagi* (9). Low

temperature crystallisation from organic solvents was employed, but an interesting, and it seems essential, feature of their procedure was the addition of a very small amount of water. With this method crystals, m.p. 5-7°, can be obtained quite easily. The material is clearly purer than that which has been prepared by distillation in a molecular still and which has until now been accepted as the purest preparation of the vitamin. Spectroscopic examination gives a value for $E_{\frac{1 \text{ per cent}}{1 \text{ cm.}}}$, 328 m μ , of 2,000, compared with the accepted value of 1,600.

Hamano continues to assert that he has prepared crystalline derivatives of vitamin A by condensation with β -naphthoyl chloride and anthraquinone- β -carbonyl chloride (10). This work stands in need of confirmation, particularly in view of the high melting points of the compounds in relation to that of the vitamin itself.¹

Assay of vitamin A.—The new value of $E_{\frac{1 \text{ per cent}}{1 \text{ cm.}}}$, 328 m μ , 2,000, affects the spectroscopic estimation of vitamin A, but this is only a small point in comparison with the other grounds for a re-examination of the whole question of the relation between the results given by chemical and physical methods for the determination of the vitamin and those obtained by direct biological experiment. The need for revising the grounds for accepting the factor 1,600 for converting values of $E_{\frac{1 \text{ per cent}}{1 \text{ cm.}}}$, 328 m μ to International Units of vitamin A was raised by Bacharach, Drummond & Morton (11) and by Ward & Haines (12), the latter pointing out that factors differing as widely as 1,100 and 2,113 were, in fact, being employed in different laboratories. Emmett & Bird (13) found evidence that the factor may depend on whether material administered in the rat test is saponified or not, there being, apparently, better absorption of esters, and therefore the derivation of a higher factor. Wilkie (14) also has criticised the factor 1,600.

A committee of the American Drug Manufacturers Association made a comparative examination of spectroscopic methods. On the basis of the accepted value of 3,000 I.U. of vitamin A for the U.S.P. reference cod-liver oil they arrived (15) at the factor 1,875.

Hume (16) reported comparable biological tests in nine laboratories on a halibut-liver oil and a concentrate prepared from it. The

¹ The writer, in collaboration with Dr. I. L. Finar, has made several unsuccessful attempts to obtain these compounds. Crystalline derivatives of sterol-like compounds have been isolated but nothing which appears to be a compound of the vitamin.

conversion factors arrived at for the oil ranged from 1,400 to 1,700, with an average of 1,470, which was accepted as being sufficiently close to 1,600 to justify continued use of the latter as the accepted figure. Lower conversion factors 1,000 to 1,200 were obtained in the examination of the concentrate, but these were attributed to its inferior stability.

More carefully controlled studies of this type are required, but meanwhile one cause of fictitious values being recorded by the spectroscopic measurement of the maximum at 328 m μ has been traced by the work of Pritchard, Wilkinson, Edisbury & Morton (17). Extracting a rich concentrate with 83 per cent alcohol they found the properties of the more soluble fraction corresponded with those of vitamin A. The less-soluble fraction showed a considerably greater biological value than would have been inferred from the reaction with antimony trichloride or from spectroscopic examination, there being a maximum at 285–290 m μ and often merely an inflection at 328 m μ . The band at 285–290 m μ had already been noted by Karrer (18) and Castle *et al.* (19), the former of whom had been inclined to regard it as an isomeric vitamin A and the latter workers as an artifact arising by decomposition of A.

Vitamin A₂.—New evidence supporting the view that more than one vitamin A exists, naturally is provided by a study of the antimony trichloride reaction. Pure vitamin A gives a blue colour with maximum at 620 m μ , but another band, at 693 m μ , is sometimes traceable. Lederer & Rosanova, examining liver oils from Russian fresh-water fishes, found only the latter (20). Further work by Edisbury, Morton & Simpkins (21) and Gillam *et al.* (22) makes it clear that this band is given by a substance of the vitamin-A class, provisionally labelled vitamin A₂, which shows bands at 350 and 287 m μ . It seems to be the chief "vitamin A" of fresh-water fish but it is also present in other liver oils.

Absorption of vitamin A and carotene.—Further work has appeared confirming the view that fats in the diet facilitate the absorption of carotene and vitamin A. Coward (23) found no significant difference between the efficiency of absorption by the rat of the vitamin A of cod-liver oil, the vitamin A and carotene of butter and the vitamin A artificially added to margarine. Her observation that the carotene of green vegetables is well absorbed is confirmed by Wilson, Das Gupta & Ahmad (24), from whose laboratory there also comes useful observations on absorption of carotene by man (25). Green

vegetables were found to be the best utilised source of the provitamin, the absorption being nearly quantitative when fats were present in the diet but only about 50 per cent when they were absent. Basu confirms the beneficial influence of fat on the absorption of vitamin A, but it is unlikely that many will share his view that the symptoms which rats exhibit when wholly deprived of fat are no more than manifestations of vitamin-A deficiency (26). The view that the reticulo-endothelial system plays a part in removing absorbed carotene and vitamin A from the blood is supported by the observations of Lasch & Roller (27). When this system is blocked by the injection of certain dyestuffs the amount of vitamin taken up by the liver from the circulation is reduced considerably. Storage in the liver in man is the subject of papers by Moore and his colleagues (28, 29, 30). Their figures, based on a large and representative amount of material, will prove of value in attempting to estimate what are the normal reserves.

A notable investigation of the minimum amounts of vitamin A and carotene required to prevent the first recognisable symptom of vitamin A deficiency, night-blindness, is described by Guilbert, Miller & Hughes (31). The importance of their observations lies in the revelation that four species investigated, cattle, sheep, swine, and rats, show approximately the same requirements, namely 25 to 30 μg . of carotene, or 6 to 8 μg . vitamin A per kilogram of body weight daily. If this relationship is found to have a wider application, and if it proves true that estimates of man's requirements come within the range, it will indicate that the vitamin is intimately concerned with the general metabolic activities of the body as a whole.

Human requirements for vitamin A.—Work is being pressed forward to ascertain the needs of man for vitamin A at different ages. It is largely based on the use of the valuable light-adaptation test devised by Jeans & Zentmire some three years ago (32). An improved form of test apparatus has been described by these investigators (33). Another simple alternative method is described by Mutch & Griffith (34), and a useful modification of the test suitable for applying to very young infants comes from Copenhagen (35). A study by Jeghers (36) shows that American students with an average intake of 4,000 I.U. of vitamin A possess normal powers of adaptation. He is inclined to add 50 per cent to this and place the daily requirements of the adult at 6,000. This is slightly more than Stiebeling's estimate of 4,200 to 5,600 (37) but several times Harris' provisional figure (38).

On the other hand, Edmund & Clemmensen found that a group of hospital nurses ingesting daily about 700 to 900 units of vitamin A a day showed powers of adaptation inside the normal range (39). This intake is less than the worst American diets studied by Jeghers. Jeghers gives a good general review of the whole question of vitamin-A deficiency in relation to night-blindness and draws attention to the practical issue whether mild night-blindness is responsible for motor accidents after dark (40).

Several papers have appeared which confirm a suspicion aroused by Jeans & Zentmire's original studies that the diets of considerable numbers of our poorer populations today are inadequate in respect to vitamin A. Maitra & Harris speak of school children in London and Cambridge (41); Aykroyd & Krishnan tell much the same story of children in India (42).

The evidence that chronic vitamin-A deficiency plays a role in the etiology of kidney and bladder stones is steadily becoming more impressive (43, 44).

VITAMIN-D GROUP

Natural formation.—Esselen, Fellers & Isgur (45) confirm the view that green plants may contain antirachitic substances but that these disappear soon after cutting and storage. Oils possessing antirachitic potency were isolated from *Sargassum* weed collected in the North and South Atlantic by Darby & Clarke (46). These materials contained, besides fucosterol, a substance showing selective absorption in the ultraviolet with a maximum at 260 m μ , and it is possible that it is related to the biological action. This is an important contribution to the much-discussed question of the origin of the vitamin D in fish tissues because, directly or indirectly, *Sargassum* weed provides nourishment for many forms of marine life.

A carefully controlled investigation by Campion, Henry, Kon & MacIntosh (47) makes it clear that the superior antirachitic potency of summer butter is due not to pasture feeding but to exposure of the cows to sun and skyshine. It is interesting, in view of the transference of vitamin D from the ration of the cow to the milk, which can so easily be demonstrated, that ergosterol added to the food in the form of an oily solution or yeast is not absorbed to any appreciable extent (48). The milk from such cows cannot be activated by radiation to a higher antirachitic potency than that of the controls, in contrast to milk to which ergosterol had been added before irradiation. The finding is in agreement with Schönheimer's proofs that

ergosterol is not absorbed from the mammalian digestive tract (49). In contrast he found that administration of ergosterol to hens resulted in an increased amount of that substance in the eggs (50). The isolation of ergosterol from egg-yolk cholesterol has been successfully achieved by Windaus & Stange (51). The nature of the provitamin in the skin has again been examined, but this time the evidence is convincing, at any rate for pig skin, because Windaus & Bock (52) actually have isolated 7-dehydrocholesterol.

Number of known "D" vitamins.—This important question still attracts considerable attention. Bills (53, 54) and his co-investigators continue to disturb our complacency by insisting that six, or perhaps as many as eight, forms of vitamin D may exist. Their examination of a number of liver oils from different species of fish shows that, rat unit for rat unit, some have about the same value as cod-liver oil in treating chicken rickets, several are less effective and a few a good deal more. It is important in the light of the work of the Göttingen school that the effect of irradiated 7-dehydrocholesterol is about the same as that of cod-liver oil, and both are significantly inferior to the antirachitic substance present in the white sea-bass liver oil. Schenck (55) has described the isolation of a vitamin D, as the 3,5-dinitrobenzoate, from fish-liver oils. It had an activity of the same order as calciferol, 40,000 I.U. per mg., and also showed selective absorption at 2,650 Å. Brockmann (56) has followed up his isolation of vitamin D₃ from tunny-liver oil by a successful separation of the same compound, as the 3,5-dinitrobenzoate, from concentrates representing no less than 150 kg. of halibut-liver oil. This examination and his experience with tunny-liver oils leads Brockmann to question whether Bills is right in thinking that fish-liver oils contain more than one form of vitamin D (57). Bills, however, has a curious flair for "spotting a winner" in vitamin-D researches and many would not be surprised to learn that his suspicions are confirmed. One such "winner," which most people failed to recognise at the time, was his demonstration that antirachitic substances were formed when solutions of cholesterol in certain organic solvents were heated with ignited fullers' earth (58). Bills has been inclined to think that the provitamin might in this case be 7-hydroxycholesterol (53). Eck & Thomas have carried investigation of the chemical activation of purified cholesterol several steps further (59, 60). The antirachitic substance formed by treatment with sulphuric acid-acetic anhydride (61) does not appear to arise from the provitamin which is present in heated purified

cholesterol. A variety of other reagents, such as sulphur trioxide, sulphoacetic acid, fuming sulphuric acid, and chlorosulphonic acid, also affect the chemical activation. Salts of cholesteryl sulphates decompose above their melting points to form antirachitic products. Cholesterilene can also be activated and the product of reaction with phosphoric anhydride is more active than that produced from cholesterol. The nature of the chemical change is undiscovered.

Clinical use of vitamin D.—In view of the impression which had gained ground in certain quarters that rickets in children is more effectively treated by natural forms of vitamin D (liver oils of cod, halibut, tunny, etc.) than by preparations of calciferol or viosterol, some importance is to be attached to large-scale clinical investigations which show that cod-liver oil has approximately the same value as irradiated cholesterol or ergosterol preparations when administered on a unit for unit basis (62, 63). The investigation of Eliot and her collaborators indicated, however, that the value of vitamin-D milk was appreciably greater than that of cod-liver oil or irradiated ergosterol. In another investigation Lewis (64) found no significant difference between cod-liver oil, viosterol, and percomorph oil, unit for unit, in a prophylactic experiment covering nearly 500 infants, but he found viosterol less effective than the oils as a remedial measure against rickets. Drake, Tisdall & Brown had found earlier that a daily dose of about 500 units of vitamin D was sufficient to protect an infant or to cure rickets (65). The new investigation tends to support that view. Mild rickets developed in a few of the infants who received only 150 I.U. daily, while the results of Lewis with dosage at the levels of 135 and 435 I.U. also support the view that good protection will certainly be afforded by ensuring an intake of 500 units.

In the opinion of the writer the clinicians have not yet given sufficient attention to the question of the relation between the intake of assimilable calcium and phosphorus and the vitamin-D requirements. There is much evidence from animal experiments that very little vitamin D is necessary providing the diet is adequately balanced in respect to calcium and phosphorus. Dols (66) reports that with 1 per cent of phosphorus and a calcium/phosphorus ratio of 3 in the diet, calcification of the bones of chicks was perfect without the intervention of vitamin D. On the other hand, when the phosphorus content of the diet was below 0.45 per cent, no amount of vitamin D produced satisfactory bone formation.

The value of Jenner & Kay's phosphatase test in the diagnosis of rickets is confirmed by a careful study of over 500 children (67). Better correlation was obtained than with analyses of serum phosphorus or estimates of the $\text{Ca} \times \text{P}$ product.

Prolonged protection against defective calcification by administration of large doses of vitamin D has been demonstrated by Rotten (68) who found that the intramuscular injection of 750 I.U. of vitamin D enabled rats to live at least fourteen months on McCollum's rachitic diet 3143 without showing bone defects. Heymann (69) has studied the rate of elimination of vitamin D by rabbits after the administration of a single dose of 200,000 units. Vitamin D remained in the liver and blood as long as twelve weeks.

Mode of action of vitamin D.—Nicolaysen has contributed important papers on this subject during 1937. His metabolic studies focus attention on the influence of vitamin D on the absorption of calcium and indicate that it probably does not directly influence the taking up of phosphorus from the gut. In calcium starvation the absorption of inorganic phosphates, glycerophosphate and the phosphorus of casein is about the same whether vitamin D is present in the diet or not. When a reduced absorption of phosphorus is noted in vitamin-D deficiency it is, according to Nicolaysen's view, due to precipitation by the increased proportion of unabsorbed calcium in the lumen of the gut. Experiments with isolated loops of intestine indicate that calcium absorption is decreased in the absence of vitamin D, although the proportion absorbed rises with concentration, whereas that of phosphorus in different forms proceeds at the same rate, irrespective of the presence of the vitamin. Vitamin D does not influence the rate of absorption of calcium from the abdominal cavity (70, 71).

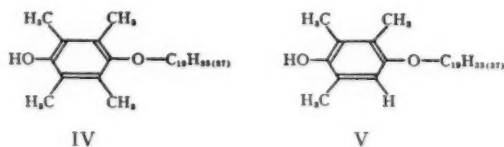
VITAMIN E

Chemical studies.—The year has seen considerable progress towards elucidating the chemical nature of the antisterility factor known as vitamin E. Drummond & Hoover (72, 73) succeeded in repeating the isolation of the allopnanate of β -tocopherol from wheat-germ oil, but obtained only very minute yields of material resembling the derivative of the α -compound as described by Evans, Emerson & Emerson (74). Todd, Bergel & Work (75) have also recorded disappointing efforts to isolate the α -allopnanate. Analyses of the parent

β -tocopherol agreed with the formula $C_{29}H_{48(50)}O_2$ advanced by the American investigators, and a study of its reactions confirms the presence in the molecule of one reactive hydroxyl group, and three, or possibly four, ethylenic linkages. The compound shows selective absorption in the ultraviolet zone with a sharp maximum at 296 m μ , recorded values of $E_{1\%}^{1\text{ cm.}}$ at this maximum being from 79 to 90. The band disappears on complete reduction with hydrogen. The second oxygen was suspected of being present as an ether linkage. The evidence from film-spreading measurements made by Danielli (cf. 73) confirmed that of Askew (76) in indicating that the area occupied by the molecule is of the same order as that of some of the polycyclic steroids.

A very important advance was made when evidence was obtained that tocopherol might be a derivative of duroquinol, tetramethylhydroquinone. Fernholz (77) isolated durohydroquinone from the products of thermal decomposition of the vitamin and McArthur & Watson (78) obtained duroquinone in making an attempt to dehydrogenate it with selenium. Fernholz regarded his observation as evidence that the vitamin is an ether of durohydroquinone, and brought forward in support of this view its strong reducing action and slight solubility in alkalis. Olcott & Emerson (79) had already indicated that the absorption band of tocopherol resembles that of hydroquinone and had again drawn attention to the curious association between anti-oxidative powers and vitamin-E activity which the work in Olcott's laboratory has revealed. They actually investigated the anti-oxidant power of α -, β -, and γ -tocopherols, but found that they did not run in the same order as the biological activity.

Drummond, Singer & Macwalter (81) had tentatively suggested that the molecule might possess an isoprenic side-chain and there was, at first, an inclination in certain quarters (78, 80) to think that the durohydroquinone isolated after heating was an artifact produced by the rearrangement of two isoprene units, but opinion is now veering round to share the view that the vitamin is a monoether of the dihydric phenol (IV).



The nature of the grouping $C_{19}H_{35(37)}$ which forms the other moiety of the molecule remains in question, although one is tempted from the film-spreading measurements, the character of the absorption bands and its relation to the sexual cycle of animals, to think that the vitamin may contain a cyclic unit, possibly with two rings, related to the C_{19} group of the sex hormones.

John (82) in examining the allophanates prepared from wheat-germ oil also obtained very poor yields of the α -compound but separated one, m.p. 147° , which has not previously been described and which on hydrolysis gave a tocopherol differing from the recognised α -, β -, and γ -compounds. On thermal decomposition it yielded cumo-hydroquinone. It is, therefore, given the provisional formula V.

It has been split by treatment with hydriodic acid to yield one fraction which showed absorption in the ultraviolet zone and one which did not.

Todd, Bergel & Work (75) find that β -tocopherol has a small optical activity $[\alpha]_D = +6$ to 7.8° , and confirm Drummond, Singer & Macwalter's (81) observation that oxidation with chromic acid yields between three and four molecules of acetic acid, indicating four, or possibly five, methyl groups.

In 1935, Kimm described the isolation from rice-germ oil of a crystalline naphthoate which on hydrolysis yielded a biologically active alcohol (83). Drummond & Hoover (73) isolated a similar naphthoate from wheat-germ oil but found the parent alcohol did not possess any action in preventing rat sterility due to vitamin-E deficiency. The inaccuracy of Kimm's work is put beyond all doubt by Todd, Bergel & Work (75) who had no difficulty in obtaining a product obviously identical with Kimm's crystalline naphthoate from rice-germ-oil concentrates. Neither this nor other naphthoates that they isolated had any biological activity.

Physiological function of vitamin E.—There has been some discussion whether vitamin E is necessary for growth. Blumberg (84) recorded stunting of young rats during the early stages of their growth on a diet deficient in vitamin E. The results of Martin (85) seemed to support Blumberg's observations, but it is not quite certain whether the form in which he administered vitamin A, carotene dissolved in ethyl laurate, is entirely satisfactory. Emerson & Evans (86) did not find that the growth curve flattened out until about the fourth month, an observation confirmed by Olcott & Mattill (87).

At this later period of development wheat-germ oil seems to exert a beneficial action on weight.

Additional evidence relating vitamin E to the pituitary gland has been presented during the year. Rowlands & Singer (88) had shown that the glands of female rats depleted of vitamin E lost some of their power to promote ovulation in the oestrous rabbit but that it was restored when the characteristic sterility had been successfully treated by giving the vitamin. Singer (89) showed, moreover, that the thyroid of the E-deficient female rat is hypoplastic compared with that of a control fed on normal diet. Injections of anterior pituitary extracts caused a slight increase in the amount of colloid, but females which had recovered their fertility by suitable feeding had thyroids similar to the hyperplastic glands seen in the normal animals. Barrie has noted that the young born of females maintained on diets containing just enough vitamin E to enable them to produce a litter are cretinous (90). She has also found that there are recognisable degenerative changes in the pituitary in E-deficiency (91). Degranulation of the basophils recalls that which is seen after castration (92), whilst it is known that degenerative changes in the acidophil cells may be related to the decreased activity of the thyroid (93). She concludes that the vitamin is necessary for the function of the anterior lobe of the pituitary gland and that the gonadotropic, galactotropic and thyreotropic hormones may be concerned. A later study of the condition in the young born of E-deficient mothers shows that immediately after birth they are normal (94). When they are suckled by a well-nourished female they grow and develop normally. If, on the other hand, they remain with their own mother the hypoplasia of the thyroid and abnormalities of the pituitary develop. This indicates that the vitamin-E content of the mother's milk is of great importance.

Muller & Muller (95) failed to trace changes in the pituitary glands of deficient females kept on E-deficient diets for as long as 240 days, but observed "castration" changes in the glands of males after a slightly longer period.

Wiesner & Bacharach (96) obtained clear evidence of diminished sexual impulse in E-deficient male rats which behaved rather like those from which the pituitary gland had been removed (97). The administration of gonadotropic hormone from pregnancy urine caused excessive development of the sex glands but did not restore the sexual behaviour. The same was true of extracts prepared from the urine

of oöphorectomised women, but a normal mating impulse was restored when a preparation from the urine of a pregnant mare was given or when an extract of anterior pituitary was administered.

Clinical trials with wheat-germ oil or concentrates of vitamin E in the treatment of repeated abortion of unexplained origin in women continue to give encouraging results (98, 99, 100). Neither Schoorl (101) nor Mason & Melampy (102) could give support to the amusing suggestion of Hill & Burdett (103) that the curious nutritive property of "royal jelly" on which queen bees are reared depends on its content of vitamin E. A dose of "royal jelly," as large as 1 gm., failed to cure an E-deficient rat.

Assay.—A timely warning against adopting a worthless "unit" proposed by Pacini & Linn (104) for vitamin-E assays is published by Bacharach (105). It is quite clear from the work of Palmer (106) and of Bacharach, Allchorne & Glynn (107) that the errors and uncertainties of the existing biological test are so disturbing that it cannot yet be regarded as suitable for quantitative studies, apart from the all-important fact that an appropriate standard is not yet available.

VITAMIN K

Chemical investigations.—Dam & Lewis have confirmed the value of distillation in high vacuum as a means of concentrating this vitamin (108). Their most potent material distils over at about 160° at 10^{-3} mm. pressure. It is an oil which withstands treatment with acetic anhydride at 100° for thirty minutes and also subjection to the action of bromine with subsequent debromination by sodium sulphite. It does not show selective absorption in the ultraviolet zone. Almquist (109) has carried investigations of his distilled material several stages further. It is rather readily destroyed by alcoholic potash; it is relevant that Dam observed that it was inactivated by adsorption on aluminium oxide. It does not show the reactions of an alcohol or ketone, but there is evidence that the molecule is unsaturated. In a later paper the distilled material is submitted to further fractionation designed to remove sterol-like contaminating substances (110). When these had been separated he found it possible to crystallise a very potent fraction at temperatures well below zero, which may be the vitamin itself. An addition of so small an amount as 2 to 4 mg. of this material to a kilogram of the basal diet prevented the appearance of the characteristic haemorrhages in young chicks, whilst 4.5 to

6.5 mg. was found curative. The active substance does not contain nitrogen, sulphur, or phosphorus, but it is tentatively suggested on very slender evidence that its molecule contains one or more benzene nuclei.

Gizzard-erosion factor.—The position is not yet satisfactorily clarified regarding the relation of the factors described by Kline *et al.* (111) and by Almquist (112, 113) as being concerned with the maintenance of a healthy condition of the lining of the gizzard in young chicks. The former group of workers think that a water-soluble vitamin is the controlling factor whilst Almquist produces equally convincing evidence that it is a fat-soluble substance.

It may, of course, be found that more than one factor is concerned. It is possible also that the discrepancies will be resolved by an explanation similar to that which after a long time has turned up to account for a curious disagreement which, in its small way, added to the confusion of the literature on pellagra. Twenty years ago Chittenden & Underhill described a condition resembling pellagra which appeared in certain of their dogs maintained on faulty diets (114). The chief symptom was a severe ulcerated condition of the mouth and tongue. The curious fact was that it was cured by the administration of carotene. It was always difficult to understand these observations after Goldberger & Wheeler had so clearly related the condition of "black-tongue" in dogs to a deficiency of one of the water-soluble B factors (115). Smith, Persons & Harvey now bring forward a satisfactory explanation (116). They have shown that in both these deficiency conditions a lowered resistance of the animal renders the mouth liable to infection with the same group of fusospirochaetal organisms so that the superficial appearance of the lesions caused by two different dietary defects is more or less the same.

The fat-soluble anti-gizzard-erosion factor described by Almquist appears to be thermolabile and readily destroyed by alkalis. It has been concentrated to some extent by freezing out inactive material from a solution of wheat-bran oil in hexane. It does not appear to be necessary for growth.

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NUTRITION*

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In this chapter an attempt will be made to review the progress in the science of nutrition under the following general headings: (a) the nutrients required by man and animals and the quantities required under different conditions; (b) the physiological effects of deficiencies of these indispensable nutrients or of their consumption in excessive amounts; and (c) the value of food materials as sources of nutrients. Since the vitamins will be considered in a separate review, somewhat less than half of the nutrition literature of the past year remains to be considered here, but even so it is obviously impossible to refer to all of the pertinent contributions, much less to discuss them critically. The success of the review will depend largely both upon the judgment of the reviewer in his selection of material and upon his diligence in surveying the many interesting publications that have appeared in the span of a year.

Before considering the original literature, reference should be made to two books on nutrition that have been published during the year, one on human nutrition and one on animal nutrition. The well and favorably known book of Sherman, *Chemistry of Food and Nutrition*, has been completely rewritten in its fifth edition. One of its most characteristic features is the emphasis and the interpretation that the author puts upon the fact, which his own experimental work has done so much to illustrate, that there are different degrees of adequacy in nutrition. This attitude has led him to formulate dietary recommendations containing, except for energy, one and one-half to four times the amounts of the various indispensable nutrients "demonstrably needed." When these recommendations are used, as they frequently are, in judging of the adequacy of the diets of selected groups of people, a depressing picture may be obtained, although no proven malnutrition may exist. If the often quoted statement that one-third of the population of the United States is ill-fed was based upon such usage, it becomes explicable, if not credible.

The textbook of L. A. Maynard, *Animal Nutrition*, will doubtless

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satisfy a long-existent demand in this field. It is mainly concerned with principles, referring the reader for facts and figures, beyond those used for illustrative purposes only, to selected readings of the nature of reviews, monographs, and original literature. It provides an inclusive, critical, and conservative survey of the present knowledge of nutrition with particular reference to farm animals.

NUTRITIVE REQUIREMENTS

Energy.—The basal requirement of energy is measured by the basal metabolism. Notable studies of the basal heat production of children have been reported by Lewis, Kinsman & Iliff (86), Talbot, Wilson & Worcester (143), Maroney & Johnston (95), and Nakagawa (115). The observations of Talbot and associates on girls indicated a closer correlation of heat production with body weight than with surface area or height. Age changes in basal metabolism, including the changes occurring at puberty, are interpreted as reflections of changes in the intensity (or speed) of growth. A number of Italian studies of basal metabolism (72, 137) testify to the extremely practical objectives of nutritional research in that country (24). Quagliariello (125) has compared the age changes in basal metabolism of Italians and Americans. At the younger ages the metabolism is more intense in Americans, but at older ages the reverse seems to be true. No acceleration in basal metabolism was noted in Italians at, or preceding, puberty. The general problem of the existence of racial differences in basal metabolism has been vigorously prosecuted by Benedict and his collaborators (13, 14, 77, 78, 105) and has been reviewed by De Moura Campos (37). The solution of the problem is complicated by the disturbing influences of climate, dietary habits, activity, and occupation and is not simplified by the possibility that racial differences in basal metabolism, if they exist, are not constant.

Maroney & Johnston (95) have assessed the total energy requirements of children varying in age from 4 to 14 years. Twenty-seven children were kept upon measured and systematically varied diets for nine and one-half months. Criteria of dietary adequacy, not in the Sherman sense of bare sufficiency but in the usual sense of full sufficiency, were normal gains in body weight and height, satisfaction of appetite, and a normal basal metabolism. The latter criterion seems well chosen since on submaintenance diets the basal metabolism of children adjusts itself to a lower level (73). The evaluation by these means of the data obtained led to the conclusion that caloric intakes

adequate for growth represented increments over the basal calories of 67 per cent for girls and 74 per cent for boys. These increments relate to growth, but are due, doubtless, largely to activity.

Studies of the basal metabolism of swine (26, 36), chickens (42), chick embryos (7), rats (17, 35), and mice (6) have been reported, with various specific purposes in view. Barbour's work (6) affords further evidence, if such is needed, of the depressing effect of under-nutrition on basal metabolism, while Deighton's investigations (36), as well as those of Davis (35) and Benedict & Sherman (17), furnish information on the effect of age. The controversy as to the best unit of size to which to reduce basal metabolism measurements still goes merrily on. It would seem difficult, if not impossible, to attempt to solve the problem within age limits where age changes are rapid. If the decision is made on the basis of measurements on adult animals, in which the only considerable disturbing factor is body size, the prospects of determining the effect of age during infancy and adolescence on the intensity of the basal metabolism would seem to be improved. The advantages of body surface as a unit of reference can be assessed only when reliable methods of surface-area determination and estimation from some easily measured dimension have been evolved for each species of animal. The tendency to use the Meeh formula with a constant determined many years ago, possibly on one animal, or even to guess at a constant as is sometimes done, affords no fair test of the surface-area law, nor does it permit valid inter-species comparisons of basal metabolism. For these reasons the paper of Marracino (96) on the surface area of sheep, and the earlier monograph by Boyd (25) on the growth of the surface area of the human body are worth-while contributions.

The effect of obesity on the basal metabolism has a bearing on the dietetic treatment of human obesity and the dietetic enhancement of the fattening of farm animals. The early work of Means (100) seemed to indicate clearly that the relation between basal metabolism and surface area in human subjects was not disturbed by obesity and since then many papers of like significance have appeared. The contrary conclusion expressed by Armsby & Fries (1) from their work on cattle must now be set aside because their method of estimating the fasting catabolism of cattle has been shown to be unreliable. Benedict & Lee (15, 16) have studied the basal metabolism of the goose before and after fattening and have found for mature geese that the increase in basal metabolism is in proportion to the increase in weight. How-

ever, when the basal metabolism per $10 W^{2/3}$ per day is plotted against body weight, the points cluster around a horizontal line (15, p. 75) at 940 kilocal. In a fat mouse weighing 59 grams, as compared with a normal mouse weighing 21 grams, the basal metabolism per gram of body nitrogen was 5.56 kilocal against 10.50 kilocal (16). These values afford no support for the protoplasmic mass theory.

Amino acids.—The productive investigations of Rose and his colleagues on the amino acid requirements of the growing rat have been continued with reports of the dispensability of glycine and serine (90), and later of cystine (159), and of the indispensability of methionine (159). An excellent summary of their work up to April, 1937, has also appeared (128). A more general summary of the nutritive significance of the amino acids has been prepared by Mazza (99).

It appears that no more than ten amino acids are required for maintenance and growth, but among these ten is one, arginine, that the body can apparently synthesize but not at a rate sufficiently rapid to meet the demands of normal growth. This unique position of arginine may mean that its value for different species of animals will depend upon the characteristic rates of growth: a slow growing species, such as man, may be able to synthesize it at a sufficiently rapid rate for all physiological demands, assuming that the synthetic capacities of the body are not closely geared to the rate of growth. The Illinois investigators distinguish essential from non-essential amino acids on the following basis: "At the present time, the distinction between an essential and a non-essential compound appears to rest solely upon the ability of the organism to accomplish its synthesis, out of materials *ordinarily available* (31), at a speed commensurate with the demands for it."

The very emphatically expressed judgment that cystine is a non-essential amino acid will come as a surprise to many, because with so many different types of dietary protein, fed at levels inadequate for maximum growth, supplements of cystine have been shown to stimulate growth. It now appears from Rose's work that diets free of cystine, except for the source of vitamin-B factors, can support rapid growth, and that the addition of cystine to such diets, in *ad libitum* feeding tests, effects no improvement in growth. Rose explains the apparent discrepancy on the assumption that "cystine is able to function in place of part of the methionine, but not as a substitute for all." To the reviewer, a more plausible explanation is

that there is a definite requirement in the growing animal for both cystine and methionine, and that both requirements can be covered by methionine, but only the cystine requirement by cystine itself. However, the evidence does not seem to be complete that rations free of methionine but containing adequate amounts of cystine are not capable of supporting growth, since in the experiments of Womack, Kemmerer & Rose (159) they may not have been consumed in sufficient amounts for this purpose. In *ad libitum* feeding experiments the production of growth is complete evidence that the ration producing it contains all nutrients necessary for growth, but failure of growth is not complete evidence of dietary inadequacy unless the amount of food consumed would, if the diet were adequate, support growth. Observations (unconfirmed, it is true) that are difficult to reconcile with the conclusion that cystine is a non-essential amino acid are: (a) the finding of Terroine, Mezincesco & Valla (145) that pigs previously brought to the endogenous level of nitrogen and sulfur excretion on a protein-free ration can store sulfur when a supplement of cystine only is given, equivalent in nitrogen to the endogenous excretion; (b) the observation of Marston (97) that dietary supplements of cystine, but not of methionine, stimulate the growth of wool on a sheep maintained on a low-protein diet; and (c) the experience of Weichselbaum (152) with a peculiar syndrome induced in rats by subsistence on the Sherman-Merrill cystine-deficient diet, recovery from which could be secured by administration of cystine, but not of methionine. That cystine and methionine may exert diametrically opposite effects in metabolism has been shown by Tucker & Eckstein (146). Consistent with Rose's conception that cystine cannot replace methionine in nutrition is the work of White & Beach (153) to the effect that methionine, but not cystine, stimulates the growth of rats on a diet containing 15 per cent of arachin as the sole protein component. The work of Block (23) on the amino acid content of brain proteins in male and female monkeys and in men and women confirms earlier work of Tadokoro and associates (141a) (on muscle proteins), to the effect that lysine occurs in greater concentration in male tissues, and suggests a corresponding sex difference in amino acid requirements.

That the amino acid requirements of animals with a simple digestive system may be different from those for ruminants has been suspected for many years on the basis that the micro-organisms of the paunch may synthesize amino acids and proteins from the non-protein

nitrogenous constituents of the feed. Such proteins would then be available for the nutrition of the host. This probability has inspired many agricultural investigators, particularly in Germany, to substitute for high-priced protein concentrates, cheaper synthetic nitrogen-containing compounds of simple structure, such as urea. Probably the most successful attempt to demonstrate that a partial substitution of protein by urea may be made, and that the urea thus included in the ration is utilized in anabolism, is that reported by Fingerling and associates (49). Working with two growing calves with a basal ration supplying sufficient energy but demonstrably insufficient protein for maximum storage of nitrogen, they were able to prove that the substitution of about 30 per cent of the protein by urea containing an equal content of nitrogen did not depress the retention of nitrogen as measured by the difference between nitrogen consumed and nitrogen excreted in urine and feces. The experiment appears to meet most of the objections that may be raised against preceding work of the same description (111, p. 571), even against that of Mangold & Stotz (92). The only possibility of serious error in the conclusions is that on the urea diet the cutaneous excretion of nitrogen may have been large enough to reduce considerably the estimated retention, as Scheunert and others (130) have observed with sheep. The importance of considering the cutaneous excretions of nitrogen, and of many of the inorganic elements with the exception of calcium and phosphorus, in critical balance studies is revealed by the recent experiments of Freyberg & Grant (55) on human subjects.

Protein.—In practical nutrition, for cogent reasons too numerous to mention here, amino acid requirements must be expressed in terms of nitrogen or of a conventional protein. If the basal heat production may be regarded as a measure of the minimum requirement of energy for the maintenance of life, then it would seem that the basal expenditure of nitrogen, the endogenous wastage of Folin, should measure the minimum requirement of protein. Terroine & Sorg-Matter (145a) and later Smuts (136a) established the fact that there is a very constant relation between the endogenous output of nitrogen and the basal production of heat, not only within any one species but among different species. Talbot (142) has shown a close relationship in children between the excretion of urinary creatinine and the basal calories per mg. of creatinine excreted, thus confirming earlier work from the same laboratory on adults. An experiment by Jackson (71) possesses the same significance. Young rats were offered free access

to a protein-free diet and were fed in addition amounts of a protein-rich mixture so regulated that weight was just maintained over a period of 15 weeks. Beginning with the second week the protein required daily for maintenance diminished steadily to the sixth week and then remained nearly stationary. The voluntary intake of the basal diet diminished also in a strikingly parallel fashion. The results are consistent with the theory that continued maintenance of young rats at constant body weight depresses the basal metabolism and, concomitantly, the endogenous protein metabolism.

Leitch (84) has published a review on the determination of the protein requirements of man in which he takes the position, quite unsupported by competent evidence, that the maintenance requirement of protein must be much greater than the equivalent in dietary protein of the endogenous loss of nitrogen. Instead of directing attention to those experiments in which minimal protein intakes have sufficed for nitrogen equilibrium, he extends consideration to all nitrogen-balance studies on adult human subjects that satisfy certain criteria of significance. From this hodge-podge of experiments in which the size of the subject and the type of dietary protein must have varied widely, since negative balances were obtained on nitrogen intakes varying from 3 to 16 grams daily and positive balances on intakes varying from 3 to 13 grams, he concludes that the minimum protein requirement for a man of average body weight is about 50 grams daily. This is equivalent in nitrogen to about three times the endogenous loss.

Many experiments on men, farm animals, and laboratory animals may be cited in favor of the theoretically sound opinion that the minimum protein requirement of the adult animal is represented by the amount of dietary protein needed to replace endogenous losses. The celebrated case of Dr. C. Röse, who lived for fifteen years on an average daily intake of about 40 grams of protein, has been repeatedly discussed in the literature and has again been presented with additional data by Strieck (140). On 24 to 29 grams of protein daily, this subject remained in nitrogen equilibrium and in a condition of excellent physical efficiency during a succession of days in which he worked on the brake ergometer to the point of exhaustion. In these experiments the cutaneous excretion of nitrogen, amounting to about 0.1 gram daily, was considered in computing the nitrogen balance. Whether this and similar experiments merely illustrate the wonderfully adaptive powers of the animal organism in meeting "dietetic insults," as von Fürth (56) thinks, or whether it represents a success-

ful attempt, against innumerable practical difficulties, to demonstrate the low minimum requirement of protein in adult man, as the reviewer believes, cannot be decided at the present writing.

The consumption of protein by adult animals in excess of the requirements for maintenance may result in a definite storage of nitrogen in the tissues (32). These stores of nitrogen are promptly available to build new hemoglobin and new plasma proteins (63) and seem to be more subject to catabolism from intoxication than are the tissue proteins (34). They are also available to supply the increased protein requirements of pregnancy in the female (132). They may be subject to hormonal control (87). They may be augmented by carbohydrate additions to a diet containing more than enough protein for maintenance (33, 81), but on discontinuing such supplements the nitrogen stores thus accrued are soon dissipated. The interval during which extra carbohydrate is able to induce the storage of reserve protein (nitrogen) is limited, with dogs, to four hours before and four hours after the ingestion of the daily meal (81). Whether these stores of labile nitrogen promote health and physiological efficiency, as well as serve emergency purposes that a simultaneous increase in dietary protein would serve as well, has yet to be demonstrated. Only when this question can be answered in the affirmative is the existence definitely established of an optimum level of protein nutrition, as distinct from an adequate protein nutrition, fully sufficient to meet demonstrable needs.

For children of increasing age the protein requirement has been assessed at 3 to 1.5 gm. per kg. of body weight daily (95). However, the method of assessment is not one that would be expected to give actual requirements, though perhaps no more critical method could be used on human subjects. These figures are three to five times the amount of protein required for adult maintenance, according to Sherman, although, as Terroine (144) has shown, the average daily storage of protein by the growing child from two to twenty years of age (1.5 gm.) is only one-fifth of the endogenous loss of protein ($N \times 6.25$). Either these estimates of Maroney & Johnston, which are quite in line with similar estimates made by others, are many times in excess of the truth, or else the child is extremely inefficient in its utilization of dietary protein.

Although it is commonly believed that muscular work has little or no effect in increasing the rate of tissue catabolism—the endogenous catabolism of Folin—Terroine and others have been critical of the

evidence offered. During the last year Mezincesco (104) has reiterated these criticisms and has presented evidence of his own, on himself as subject, in support of the view that muscular work does increase endogenous nitrogen metabolism taken as the sum of urinary and fecal nitrogen on a low-nitrogen diet. The urinary excretion of nitrogen was so irregular as to obscure any small effect of work that might otherwise have been evidenced. The sum of urinary and fecal nitrogen seemed to be at a higher level during the work days than before, but this increase was entirely in the fecal excretion, apparently in response to the greater intake of food. This would indeed be a very indirect effect of work.

Fat.—Interesting suggestions have appeared in the literature to the effect that human subjects need certain unsaturated fatty acids preformed in the diet, as does the rat according to the well-known work of Burr and associates. Brown & Hansen (27) have reported that the percentages of arachidonic and linolic acids in the fatty acids of blood serum are lower in eczematous human subjects than in normal subjects and that they increase on convalescence. However, Brown, Hansen, and others (28) also report that a normal human subject was able to subsist for six months on a diet providing only 2 grams of fat daily. Fed to rats, this diet produced symptoms of fat deficiency in the usual length of time. Although the iodine number of the serum lipids decreased, as did also the content of arachidonic and linolic acids, no pathology was noted in urine, blood, or basal metabolic rate and the subjective findings were favorable. There is no sure indication in any of these observations that humans need certain unsaturated fatty acids, but if they do it seems incredible that on any practicable diet a deficiency of such acids would occur. In this connection Turpeinen (147) has shown, by the usual *ad libitum* feeding technic, that arachidonic acid is a curative agent in rats for fat-deficiency symptoms, being more potent even than linoleic acid. Hansen & Brown (61) have observed a decrease in the unsaturation of serum lipids in rats subsisting on a fat-free diet. Both linolic and oleic acid administration increased the iodine number of the total lipids, although only the former effected a complete clinical cure of the deficiency symptoms.

Minerals.—The presence of bromine in animal tissues (41) raises the question of its indispensability in nutrition. Winnek & Smith (157) have been able to raise rats successfully on a synthetic diet containing less than 0.5 part per million with no evidence that bromine

is an essential nutrient, thus confirming previously expressed opinions (41, 148). The contention of Beynon (19) that copper is not an essential element in nutrition, in spite of a mass of evidence published to the contrary, can hardly be judged on the basis of the incomplete report submitted.

The indispensability of cobalt in nutrition has been strikingly confirmed (150). The extremely small requirement of the element, judged from the size of the doses curing the deficiency disease, is quite remarkable. Filmer & Underwood (48) found that 0.01 mg. of cobalt daily exerted no appreciable effect in curing enzoötic marasmus in sheep; 0.03 to 0.05 mg. was appreciably effective, while 0.1 mg. was approximately the lowest daily dose that would induce normal growth and health on cobalt-deficient pastures. Doses of 0.3 to 1 mg. of cobalt daily appeared to be sufficient for cattle. Askew & Dixon (2) reported that weekly doses of 0.29 mg. of cobalt were effective in controlling the lamb sickness known as Morton Mains disease.

The maximum retention of a mineral element by a growing animal is a measure, unaffected by the source of dietary minerals, of the requirement of that mineral for growth, though only when the mineral stores are saturated (47). Proceeding on this basis Stearns & Stinger (139) have studied the iron retention of human infants. From studies of the iron metabolism during the ingestion of varying amounts of iron as food iron or as soluble salt, it appears that an intake of 0.5 mg. per kg. of body weight is essential for equilibrium and that intakes of 1.0 to 1.5 mg. per kg. permit retentions averaging 0.33 mg. per kg.; this represents a gross utilization of approximately one-fourth of the dietary iron. For the first six or eight weeks after birth an infant may be expected to lose iron from its body at an average daily rate of about 1.25 mg. (138), coincident with destruction of blood hemoglobin, but the total iron thus lost is only a fraction of the iron liberated from hemoglobin, so that a storage of iron is occurring simultaneously with the exhibition of negative balances.

In a report of iron-balance studies on four young women, extending over three to six consecutive menstrual cycles, Leverton & Roberts (85) never observed negative balances when the intake of iron was not less than 0.225 mg. per kg. per day. The iron requirement of these subjects was assumed to equal the iron losses in feces, urine, and menses, and averaged 0.206 mg. per kg. of body weight daily. This assumed requirement is too high if the feces contain any unabsorbed dietary iron, or may be too high if, as in three of the four

subjects in this study, the subject is storing iron. The "optimal allowance" of iron is placed at 50 per cent above the requirement, a "custom" that cannot be criticized because admittedly it is based neither on evidence nor on ratiocination.

Brull (29) has reported fourteen mineral balance studies of three days each on four adult convalescing hospital patients apparently free from nutritional disturbances, who were subsisting upon a diet adequate in all respects except that it was low in calcium, phosphorus, and magnesium. The subjects were continually in negative phosphorus balance; they were in negative calcium balance in all but one period and in negative magnesium balance in all but five periods. The requirements were computed, according to the usual plan, from the total excretion; per kg. body weight per day they averaged 8.2 mg. for calcium, 13.9 mg. for phosphorus, and 3.4 mg. for magnesium, with considerable variation among individuals. The calcium requirement for a 70 kg. adult amounts to 0.57 gm. daily, a figure somewhat higher than the Sherman estimate but practically identical with that of Leitch (83), obtained by his unique method.

In the preparation of synthetic diets the proper salt mixture to use is often a puzzling problem. The introduction of a new salt mixture (70) may not clarify the situation unless it is shown to be of superior value. However, that suggested by Hubbell, Mendel & Wakeman can hardly be said to have been adequately tested by growth and calcification studies only, and its failure to contain cobalt and zinc may be urged against it if it may be fairly concluded that these are essential minerals.

A summary of available information on the mineral nutrition of farm animals, including attempts to estimate calcium and phosphorus requirements by an analytical or factorial method, has been published by Mitchell & McClure (114).

Before leaving the subject of nutritive requirements, attention may well be given to an idea expressed by Szent-Györgyi at the meeting in September, 1937, of the Royal Academy of Italy in Rome, devoted to a consideration of the present state of our knowledge of nutrition. Szent-Györgyi argued that nutritive requirements determined in as favorable environments as can be obtained—which is according to the usual procedure—may be too low. The fact that animals appear to be in optimal condition in a "protected environment" is not sufficient proof that they are, in fact, in optimal nutritive condition. They must be subjected to adverse conditions which induce

a severe physiological strain—infection, toxins, low or high temperatures, high humidities—in order to determine whether a more generous supply of the essential nutrients will permit a more successful physiological adjustment. Somewhat in support of this idea is the work of Miller & Rhoads (106) which shows that the symptoms of black-tongue in dogs appear much sooner when the dogs are also given amidopyrine, even in doses without effect on adequately fed animals.

PHYSIOLOGICAL EFFECTS OF NUTRIENT DEFICIENCIES AND EXCESSES

Dietary deficiencies may give rise to a generalized under-nutrition or to specific symptoms more or less pathognomonic of the dietary fault. Thus, a deficiency in energy intake leads to emaciation, but also to a reduction in basal metabolism (73). Amino acid deficiencies rarely induce specific lesions, though a lack of valine, according to Rose (128), brings about a sensitivity to touch in experimental rats and a severe lack of co-ordination in movement, symptoms readily cured by administration of valine.

Protein deficiency.—Rations practically free of protein may be fed to pigs or rats for several weeks with only moderate losses in body weight, but in the case of sheep Miller (107) has reported digestive disorders occasioned by such a diet. This outcome could be avoided simply by the substitution of casein for starch. Hoelzel & Da Costa (67) observed ulceration of the stomachs of rats and mice and ulceration of the duodenum of mice as a result of the continued ingestion of diets deficient in protein, a condition the appearance of which was accelerated by low room temperatures.

Low-protein diets will produce hypoproteinemia (101, 120) in which the serum albumin suffers the greatest decrease, hemoglobin next, and serum globulin least, on the basis of quantities originally present (151). But, on the basis of absolute amounts removed, the loss in hemoglobin is six times as great as that of serum albumin which in turn is seven times as great as that of serum globulin. Melnick & Cowgill (101) believe that, while deficiency of dietary protein may be a significant factor in the production of hypoproteinemia, some other factor, "such as an impairment of or injury to a specific mechanism responsible for the formation of serum protein, may play an accessory, if not the primary, role . . ." Apparently as a direct consequence of the hypoproteinemia and reduced osmotic pressure,

induced directly or indirectly by a low-protein diet, an edema of the tissues develops (89).

The consumption of diets low in protein and high in fat will induce excessive storage of fat in the liver, a condition apparently preventable by various dietary agents (91). The solution of this problem surely involves much more in the way of food control than is currently being exercised.

Protein excess.—Blatherwick & Medlar (22) have produced chronic nephritis in young rats on diets containing 75 per cent of liver, functional disturbances of the kidney appearing before histological changes indicative of nephritis became apparent. Diets containing 75 per cent of casein were considerably less toxic. The authors admit that "if the harmful substance exists in the diet, it may not necessarily be confined to the protein fraction." The toxicity of high-gliadin diets has been proven for dogs, but not for rats, by Melnick & Cowgill (103), the convulsive symptoms produced suggesting protein sensitization. In an attempt to produce atherosclerosis in rabbits by feeding diets containing 33 and 38 per cent of protein from vegetable sources, Freyberg (54) obtained only negative results. These results suggest that the atherosclerosis observed many years ago by Clarkson & Newburgh (30a), when rabbits were fed diets rich in muscle meat, was induced by non-protein constituents of these animal foods.

Fat.—It has been claimed by Burr & Beber (30) that rats in a condition of fat deficiency exhibit an elevated metabolic rate as compared with normal controls. However, the observed differences, which seem real enough, are impossible to interpret because no attempt was made to minimize activity by exposure of the animal to light; also, in the feeding periods, the deficient rats were allowed to consume more food than their controls. These two factors, rather than any inherent disturbance of energy metabolism in "fat-deficiency disease," may have been responsible for the observed differences in metabolic rate.

Minerals.—Despite the participation of calcium in many important physiological functions, dietary deficiencies of calcium have rarely been observed to produce specific deficiency systems (114). This is probably because the daily need for calcium in the active tissues of the body is so small compared with the immense stores in the skeleton. However, Martin (98) has reported that dogs fed a diet containing only 30 p.p.m. of calcium will develop, after many weeks, a

characteristic syndrome terminating in tonic-clonic convulsions in spite of the fact that the serum-calcium concentration is maintained within normal limits.

The results of a dietary deficiency of phosphorus are much more severe and appear much sooner than those of a deficiency of calcium. Severe anorexia and impaired growth appear early, and the inorganic phosphorus of the blood serum drops to very low levels (79). In the female, estrus is disturbed and the basal metabolic rate is depressed. However, when the food intake of control animals is lowered to equal that of the phosphorus-deficient animals, the basal metabolic rate of comparable animals is not significantly different (58). As Goss & Kleiber observe: "The results emphasize again the importance of controlling the food intake by paired-feeding in metabolism studies on deficient diets . . ." The failure of Forbes (50) to detect any appreciable effects of phosphorus deficiency except in the extent of phosphorus storage would seem to be attributable to the fact that a sufficiently low level of dietary phosphorus was not tested, the low-phosphorus rations used containing 0.133 and 0.137 per cent of the element. The remark that "normal growth could not have been obtained on a diet still lower in phosphorus" is probably true, but the attainment of normal growth in experimental animals is generally sufficient evidence that a dietary deficiency has not been imposed.

Hoobler, Kruse & McCollum (68) have continued the studies of the Johns Hopkins group on magnesium deficiency, using dogs as subjects. A ration containing 2 p.p.m. of magnesium ultimately induces vasodilatation and a subsequent heightening of nervous irritability which terminates invariably in a convulsive seizure. A pronounced drop in serum magnesium appears to be the immediate cause of nerve involvement. In rats, hyperemia and purpura of the skin (59, 131) followed by nutritional failure, cachexia, and kidney damage are also observed on a low-magnesium diet. The action of magnesium carbonate when added to the diet of rats in relieving experimentally-induced hypertension is an interesting observation made by Rubin & Rapoport (129).

Sodium deficiency in rats leads to retarded growth, marked disturbances of the eye and of the reproductive functions, changes in the adrenal glands, and alopecia (117). Forbes and associates (74), using a diet containing 3.5 times the concentration of sodium on which the above symptoms were observed in eight weeks or less of feeding, observed no symptoms after ten weeks of feeding a diet containing

0.007 per cent of sodium, although indications of impaired utilization of feed and subnormal sodium content of tissues were obtained. Excess of dietary sodium, either as chloride or as sulfate, in an otherwise well-balanced diet has been observed by Lecoq (82) to induce polyneuritis.

The symptoms of potassium deficiency in rats have been described by Schrader, Prickett & Salmon (131) to consist of lethargy, abdominal distension, edematous kidneys, cyanosis, failure of growth, and early death.

A dietary deficiency of iron or copper or both, as in exclusive milk feeding, will produce a nutritional anemia in young growing animals. Human infants are susceptible to this type of anemia and respond maximally either to iron or to iron and copper (44) therapy depending apparently on the nature of their diet. Manville & Chase (93) have found that young rats on an anemia-producing diet become progressively less able to withstand low temperatures as the severity of the anemia increases. Wolf & Tschesche (158) claim that rats in a condition of milk anemia may be used, with a cautious interpretation of results, in assaying liver extracts for anti-anemic activity, but it is hardly conceivable that such assays would possess any significance for pernicious anemia. In adult hemorrhagic anemia (idiopathic hypochromic anemia) iron therapy is effective, and neither copper nor liver extracts confer any additional benefit (51). The massive doses of iron needed for effective treatment suggest that trace elements, particularly cobalt (149), may be the effective agents.

A naturally-occurring copper deficiency among sheep in certain areas of Australia has been reported by Bennetts & Chapman (18). This condition, known as "enzootic ataxia," is characterized by anemia of the mother ewes and ataxia in the progeny. Copper supplements to the ration will prevent and cure both disorders.

A number of deficiency diseases in cattle and sheep, previously suspected of being iron deficiencies, have been shown to be prevented and cured by very small doses of cobalt. These diseases are characterized in general by anorexia, emaciation, and anemia, with histological changes and lesions in muscle, spleen, and liver demonstrable on post-mortem examination (116). Such diseases have been reported in restricted areas of Australia, New Zealand, and Florida and have been variously called "enzootic marasmus" (48), "bush-sickness" (2), "Morton Mains lamb ailment" (2, 40) and "salt sick" (48). In connection with studies of these fortunately restricted livestock ailments,

interesting investigations of cobalt metabolism have been made (4, 12, 39), with indications that the cobalt content of the liver may be diagnostic (3). Neither the cobalt content of soils (75) nor of forage (116) can be relied upon in diagnosis, because of the minute amounts of the element present: in fact, spectrographic analysis has not demonstrated the occurrence of cobalt in forage known to have an adequate concentration (116).

In chickens, a bone disease known as "slipped-tendon" or perosis, which occurs in battery-fed birds, has been shown to involve a manganese deficiency in the ration (64, 154), although other factors may also be involved. In perosis there is gross enlargement and malformation of the tibial-metatarsal joint, with displacement of the gastrocnemius tendon from its condyles. Birds completely crippled in this fashion soon die. The condition is prevented or cured by the addition of manganese salts to the ration or of manganese-rich feeds, such as rice bran. Laying hens subsisting on a perosis-producing diet will yield eggs of low hatchability because of abnormal development of the embryos; the deformity produced (88) is a chondrodystrophy. Embryos dying of chondrodystrophy contain subnormal concentrations of glycine (121).

Further work on the physiology of zinc in rats, in which the effects of feeding low-zinc diets were observed, have been reported by Hove, Elvehjem & Hart (69). Some indications were obtained that on zinc-deficient diets intestinal absorption is impaired, but the evidence is not impressive, and the postulated relationship of zinc and pituitary hormones seems unconvincing since pituitary transplants were not made in rats normally fed, as well as in rats on the zinc-deficient diet. The relationship of food intake to gain in body weight is assigned a significance that it hardly deserves, though this is not an uncommon error in nutrition reports.

The effects of diets deficient in all salts upon the intestinal flora (46), the respiratory metabolism (80), the composition of the tissues (45), and intestinal motility (126) have been reported. The significance of results of multiple dietary deficiencies seems difficult to assess since their explanation cannot be given in terms of definite nutritive factors. The addition of individual minerals to such salt-deficient diets would seem to be a less effective experimental procedure in studying mineral functions than the withdrawal of minerals one at a time from an otherwise complete diet.

Dennig's (38) observations of a favorable effect of alkaline salt

additions to the diet on the performance of muscular work is a contribution to an age-old problem. The finding of French & Bloomfield (52) that rats once subjected to dietary deficiency are more susceptible to its effects, judged by loss of weight only, in a subsequent period of defective feeding is interesting though inexplicable. *

UTILIZATION OF FOOD NUTRIENTS

Energy.—In a number of nutrition laboratories the effect of dietary deficiencies on the utilization of food energy has been a prime subject of interest. Kleiber, Goss & Guilbert (79) showed that phosphorus deficiency in beef heifers lowered the utilization of metabolizable energy. Continuing a series of studies of this character, Forbes and his group have investigated during the past year the effects of deficiencies in phosphorus (50), sodium (74), and iron and copper (20) in growing rats using the paired-feeding technic. The phosphorus experiment was negative, apparently because a sufficient degree of phosphorus deficiency was not imposed. The experiments involving deficiencies of sodium and of iron and copper indicated significantly smaller energy retentions for the deficient rats than for their pair-mate controls and significantly larger total productions of heat. While in all probability these excellently controlled experiments indicate an impairment of energy utilization by the imposed dietary deficiencies, no conclusions to that effect were deduced by the investigators themselves, possibly because the experimental results may be interpreted also as indicating merely a difference in the disposal of utilized energy between storage and muscular activity. Hamilton (60) has made a preliminary report of an extensive investigation on growing rats, proving beyond a doubt that a deficiency, and also an excess, of dietary protein impairs the utilization of food energy by increasing the calorogenic (specific dynamic) effect.

Protein.—Many experiments on the utilization of dietary protein have been reported in the year's literature, mainly by the nitrogen-balance method designed to permit the calculation of a biological value in the Thomas-Mitchell sense. An exception is the work of Markuze (94) on cereal products, the results being expressed as ratios of gain in body weight to protein consumed. In this investigation the low value for millet proteins is noteworthy, although the disturbing effect of a variable intake of food on the ratio computed complicates the interpretation.

The biological values of a number of Indian cereal, legume, and

vegetable proteins were measured by Swaminathan (141), and of the proteins in two varieties of rice by Basu & Basak (8). Aman and Aus rice gave practically the same biological values, but in growth experiments the Aman-rice proteins at a 5 per cent level permitted growth and a gain of more than 2 grams per gram of protein consumed—surprising for such a low level of dietary protein. Aus-rice proteins at the same level did not even support maintenance until supplemented by either cystine or methionine (9). Basu, Nath & Mukherjee (10) investigated the proteins of three legume seeds; cooked soybeans gave a lower biological value than raw, contrary to a number of previously published data. Other experiments on hegar proteins and their supplementary relations (136), nut proteins as compared with those of beef (109), and lactalbumin and casein (76) have contributed data to this important subject. In the last-mentioned experiment the superiority of lactalbumin over casein was clearly shown except when casein was supplemented with cystine. Henry, Kon & Watson (66) observed no impairment of the biological value of milk proteins through pasteurization.

Ganchev & Popov (57) have reported on the biological values of a number of Bulgarian protein concentrates used for pigs, while Miller, Morrison & Maynard (108) have presented additional evidence that marked differences in the biological values of feed proteins are not to be expected with ruminants (sheep), probably because of the intermediary action of paunch micro-organisms.

Melnick & Cowgill (102) have made a very careful comparison with dogs of the value for maintenance of lactalbumin, serum protein, casein, and gliadin, while Robscheit-Robbins & Whipple (127) have shown that in hemoglobin regeneration after hemorrhagic anemia, the globin moiety of hemoglobin is much better utilized by dogs than liver proteins. Pearson, Elvehjem & Hart (123) have made a similar study of the value of dietary proteins in promoting hemoglobin regeneration in milk anemia of rats, but the basal diet was fed *ad libitum*. The experiments of Pearson and associates (122, 124) on the relation between protein quality and the regularity of estrus are of interest.

Minerals.—The favorable effect of lactose on the utilization of calcium has again been confirmed (113, 119). French & Cowgill (53) have shown with dogs, and less certainly with rats, that this effect is confined to the immature animal. Sucrose and fructose exert a more favorable effect on calcium utilization than does glucose

(113). The high utilization of the calcium of inorganic salts by growing swine, retentions ranging from 50 to 70 per cent of intakes (110), are sharply contrasted with a retention of 20 per cent of milk calcium by pre-school children reported by Outhouse and associates (118).

The practical importance of determining whether the pasteurization of milk impairs its nutritive value in any way has inspired a large amount of experimental work. When raw and pasteurized milks are fed as the exclusive diet to experimental animals (133), or in amounts not shown to provide insufficient quotas of the nutrients being studied (21, 155, 156), it seems impossible to interpret the experiments when no differences are observed, since super-adequate nutrition may obscure small degrees of nutritive impairment. The carefully controlled experiments of Henry & Kon (65) and of Auchinachie (5), in which all precautions necessary to the attainment of significant results were observed, revealed no impairment of calcium utilization; these findings are in opposition to the earlier results of Ellis & Mitchell (43) who used essentially the same degree of control.

Suggestions for improving the technic of determinations of the availability of food iron in hemoglobin regeneration from milk anemia have been proposed by Smith & Otis (134, 135) and by Mitchell & Hamilton (112). The suggested improvements relate in particular to the recognition of sex differences, to the greater depletion of iron stores by the addition of copper supplements to the depletion diet of milk, and to the control of the intake of the basal milk diet during the experimental period. The advisability of using glass cages in rat-feeding work of this description is not by any means a closed book. Such refinements will increase the accuracy of work of this character and seem necessary to the solution of such problems as the determination of all the nutritive factors in liver involved in hemoglobin formation (62).

Beard & Boggess (11) have submitted additional convincing evidence that injected iron (colloidal) is much better utilized for hemoglobin formation than is iron administered *per os*.

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THE BIOCHEMISTRY OF MUSCLE*

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The chemical composition and architecture of muscle.—Bell (1) has continued his studies on the molecular structure of muscle glycogen (horse muscle), using for the first time the "end group" assay method. The results show that the glycogen molecule is built up of chains of eleven to twelve glucose units. Its properties do not distinguish it from the twelve-unit liver glycogens so far examined; this is consistent with the results of a comparison of liver and muscle glycogen carried out by Young (2) in which no differences in properties could be demonstrated.

Riesser (3) has demonstrated rather large daily variations in the total phosphorus content of guinea-pig muscle. The cause of these variations is so far unknown. A certain relation between the phosphorus content and meteorological conditions is suggested by the author.

Parschin (4) has studied the non-protein nitrogenous compounds in dog muscle. The presence of carnosine, anserine, creatine, creatinine, methylguanidine, and carnitine was demonstrated.

Baldwin & Needham (5) have made a comparison of the distribution of phosphorus in the electrical tissue of *Torpedo* and in muscular tissue and have demonstrated that the resemblance between the two tissues in this respect is very close. It was possible to demonstrate that a series of enzymatic reactions known to take place in muscle extracts proceeded also in extracts from the electrical tissue.

Further evidence of the presence of both phosphocreatine and phosphoarginine in echinoid jaw muscle was obtained by the demonstration that extracts of echinoid muscle contain enzymes capable of bringing about the synthesis of both phosphoarginine and phosphocreatine by the usual series of reactions. Extracts of unstriated holothurian muscle can synthesize phosphoarginine only, and this phosphagen alone is present in the holothurian muscle. The investigation on the distribution of phosphagen in the Echinodermata was extended to include the Crinoidea and the Ophiuroidea. In the former only phosphoarginine was found, and in the latter only phosphocreatine. This observation seems to be of considerable interest as additional

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evidence in favour of the theory of the evolution of vertebrates from some echinoderm type through a primitive enteropneust type.

Moore & Wilson (6) state that extracts from the adductor muscle of *Pecten magellanicus* (purchased in the market) contain no arginine but octopine, a compound which, it is suggested, is a derivative of arginine formed by the substitution of the α -amino group with propionic acid attached through its α -carbon atom. Both arginine and octopine have been isolated from extracts of quite fresh tissue.

Dworaczek & Barrenscheen (7) have determined the organic phosphorus compounds in smooth muscle. They find phosphocreatine in only small quantities, but, judging from determinations of creatine, they are inclined to think that the intact tissue may contain much more phosphocreatine. Adenosinetriphosphate was present in considerable quantities. This compound was isolated as the acridine salt according to the method of Wagner-Jauregg (8), and its identity with the adenosinetriphosphate of skeletal muscle was established. Hexose-phosphate was present in only insignificant quantities, but, as incubated muscle pulp shows a marked increase in total acid-soluble phosphate, smooth muscle must contain rather large quantities of acid-insoluble phosphorus compounds (phospholipoids, phosphoproteins, and nucleic acids).

Based on determinations of the molecular weight, Lohmann & Schuster (9) conclude that adenylypyrophosphate is a simple nucleotide and that Ostern's diadenosinepentaphosphate must be a mixture of adenosinediphosphate and adenosinetriphosphate. At this point it may be mentioned that Lohmann's formula for adenosinetriphosphate (adenylypyrophosphate) is still under discussion. Barrenscheen & Jachimowicz (10) claim that bone phosphatase is able to liberate the acid-stable phosphate group without affecting the acid-labile groups, a finding which is inconsistent with Lohmann's formula.

Hastings & Eichelberger (11, 12, 13) have carried out a series of investigations on the exchange of salt and water between muscle and blood. Average normal values for sodium, chloride, water, potassium and total base in dog serum and skeletal muscle were determined. Based on the assumption that all of the muscle chloride is in the extracellular phase, this phase is calculated to amount to a maximum of 17 per cent of the muscle weight. The changes in intra- and extracellular water were determined under different experimental conditions. One particularly interesting finding is that in dehydration (injection of hypertonic solutions) a decrease in muscle volume may be

due to a marked shrinking of the muscle cells accompanied by an increase in the extra-cellular phase. A calculation of the extra-cellular phase in muscle from the muscle chloride and the chloride concentration in plasma has also been used by Fenn & Goettsch (14) in experiments in which they have demonstrated a considerable increase in extra-cellular water in nutritional muscular dystrophy. The validity of such a calculation has been further established through experiments by Eggleton, Eggleton & Hamilton (15). Isolated frog muscles were soaked in Ringer solutions of varying tonicity and varying chloride concentrations. The experiments led to the conclusion that the chloride present in such muscles is contained in only one quarter of the muscle and is free to diffuse to or from the surrounding medium. In the body only 10 to 15 per cent of the muscle substance is occupied by chloride. The cause of the increase of "interspace" in a soaked or perfused muscle is at present unknown.

Muscle proteins.—Mirsky has continued his investigations on myosin. In experiments on the effect of heat on myosin isolated from frog muscles it was found (16) that heat denatures myosin in two distinct steps, one of which can be identified with thermal contraction (the reversible heat shortening of muscle) and the other with thermal rigor (the irreversible heat shortening). The first step of heat denaturation, occurring at 37°, is characterized by a high temperature coefficient and no change in the number of free sulphhydryl groups; the second, occurring between 40° and 45°, has a much lower temperature coefficient and is accompanied by a marked change in sulphhydryl groups.

Isolated myosin was shown to coagulate by freezing and by drying (17). From differences in solubility after coagulation, and from the fact that coagulation by drying is not accompanied by an increase in the number of sulphhydryl groups, it appears that this coagulation is a distinctly different phenomenon from the coagulation produced by such agents as heat and acid.

The coagulation of myosin was also studied in frozen powdered and dried muscle substance (18). If a quantity of water equal to that previously removed were added to the dry muscle powder, the myosin in it instantly coagulated. The coagulation is a very rapid process at 20° (Q_{10} , approximately 2). It readily occurs in the absence of free calcium. If a large volume of water were added to the dry muscle powder, coagulation did not occur. Addition of a large volume of dilute saline, however, brought about coagulation. If

myosin were extracted from the dry muscle powder by adding a concentrated salt solution, and this solution were then diluted by adding water, no coagulation occurred, though the same volume of dilute saline added directly to the muscle powder would have brought about coagulation. From this observation the conclusion is drawn that the myosin must be in its proper place in the structure of the muscle fibre if rapid coagulation is to occur. The difference in the behaviour of isolated myosin and myosin in dry muscle powder is, it is suggested, due to a slight enzymatic hydrolysis taking place during the extraction.

The demonstration of a coagulation of myosin accompanied by only a very slight change in the configuration of the protein molecule, and of the extreme lability of the myosin "in situ," justifies the author's interesting discussion of the probable relationship between myosin coagulation and contraction. The author quotes Deuticke (19) by saying: "Deuticke (1930) has observed that myosin loses its solubility after a normal contraction," and considers Deuticke's observation as evidence that "the change in myosin which takes place on wetting the powder is substantially the same as that which occurs in contraction." This quotation, however, is apt to mislead, as the changes in solubility observed by Deuticke were brought about by prolonged stimulation and consequently were probably more closely related to fatigue than to the "mechanism of contraction."

Using the amount of phenolindo-2,6-dichlorophenol reduced by a known weight of protein as a measure of the free sulphydryl groups, Todrick & Walker (20) find that native myosin contains free sulphydryl equivalent to 0.27 per cent cysteine. This figure agrees with that found by Mirsky. In contrast to Mirsky, however, Todrick & Walker state that the number of free sulphydryl groups does not change after heat denaturation.

Bailey (21) has studied the composition of the myosin and myogen of skeletal muscle. The total nitrogen, amino-nitrogen, total sulphur, cystine, methionine, tyrosine, and tryptophane of a series of myosins from skeletal muscle were determined. Mammalian and avian myosins have a uniform composition. Rabbit myogen differs markedly in composition from rabbit myosin. In myosin, methionine is elaborated in preference to cystine (methionine 3.50 per cent, cystine 0.72 to 1 per cent).

Based on investigations of the thermoelastic properties of muscle, Meyer & Picken (22) conclude that "The elastic system of the

muscle behaves as if composed of two components, flexible protein chains forming a three-dimensional network, and free chains in the meshes of this net."

The mechanism of anaërobic metabolism.—The Embden-Meyerhof scheme of glycolysis has not been altered in the past year. Investigations of Meeraus & Lorber (23) on extracts of smooth muscle and of Ochoa (24) on extracts of heart muscle indicate that the glycolysis of these types of muscle involves the same intermediate steps as those known from the glycolysis of skeletal muscle.

During the past year the nature of the cozymase action in glycolysis has received a chemical explanation. In experiments closely corresponding to those of Warburg & Christian (25) on the fermentation system, Euler and coworkers (26) demonstrated that hydrogen is transferred from triosephosphoric acid to pyruvic acid through the diphosphopyridinenucleotide (cozymase). Through this demonstration the general significance of Warburg's pyridine catalysis in biological oxido-reductions has been definitely established. Moreover, Meyerhof, Schulz & Schuster (27) were able to show that the transfer of hydrogen through the diphosphopyridinenucleotide only takes place if at the same time a transfer of phosphate through the adeninenucleotide takes place. The enzymatic oxido-reduction: triosephosphate + cozymase = phosphoglycerate + hydrocozymase, does not take place if the adeninenucleotide does not transfer phosphate from inorganic phosphate to a phosphate acceptor. The phosphate acceptors of alcoholic fermentation are glucose or hexosemonophosphate (Warburg's fermentation test); the phosphate acceptor of muscle glycolysis is creatine. The adeninenucleotide changes between the states: adenosinetriphosphate \rightleftharpoons adenosinediphosphate. Ohlmeyer & Ochoa state that the phosphate transfer is stimulated by manganese ion (28), but inhibited by sodium ion (29). Ohlmeyer & Ochoa claim that in the presence of manganese ion the cozymase is also able to transfer phosphate, whereas Euler and coworkers (30) are of the opinion that the observations of Ohlmeyer & Ochoa are due to a setting free of small amounts of adenylypyrophosphate from the cozymase, corresponding to the alkali inactivation of cozymase.

According to investigations by Kendal & Stickland (31) it seems that yet another glycolysis coenzyme factor exists. Minced muscle was washed repeatedly with potassium chloride solution and then extracted four times with disodium hydrogen phosphate solution,

which is specially suitable for freeing the apoenzymes. The fourth phosphate extract was completely free of coenzymes but still rich in apoenzymes (apomyozymase). Whereas the third phosphate extract could still be activated by the coenzymes known (cozymase, adenine-nucleotide, magnesium ion, and traces of hexosediphosphate), the fourth phosphate extract could not be so activated. The latter could only be activated by boiled muscle extract, which evidently contains still another essential and unidentified coenzyme. The new factor, which is not identical with the zymohexase, is resistant to boiling but not to ignition; the authors conclude from this that the unidentified factor is organic in nature.

The phosphorylation of glycogen or starch (32) takes place in two different manners: (a) a direct transfer from adenylypyrophosphate (33); (b) esterification by inorganic phosphate (34). According to the paper of Lehmann & Needham, the esterification by adenylypyrophosphate is faster than that by inorganic phosphate. According to Kendall & Stickland (35) the esterification by inorganic phosphate requires magnesium ion and traces of adenine-nucleotide. In the system of Kendal & Stickland the phosphate cannot pass through the adenine-nucleotide because a phosphorylation of adenine-nucleotide demands an energy-yielding reaction, and no such reaction is possible in this system. The esterification therefore takes place directly from the inorganic phosphate, a process previously studied by Parnas (36). As the adenine-nucleotide acts in concentrations down to $5 \times 10^{-6} M$ (induction effect?) it is scarcely possible by means of the usual dialysis methods to reduce the concentration of adenylic acid or inosinic acid below the active level. As Parnas (36) admits the impossibility of obtaining an absolutely coenzyme-free system by dialysis, there is no disagreement between Parnas and Kendal & Stickland.

By means of the above-mentioned method of Kendal & Stickland (repeated extraction with disodium hydrogen phosphate solution) it is possible to get an enzyme solution completely coenzyme-free, the so-called apomyozymase (fourth phosphate extract). Esterification of starch in this system yields principally pure glucose-1-monophosphoric acid (Cori-ester).

The important phosphorylation of adenylic acid by inorganic phosphate has been minutely investigated by Needham & Pillai (37). In agreement with the researches of Dische (38) on haemolysate, they show that the phosphorylation of adenylic acid depends on the

oxido-reduction between triosephosphate and pyruvic acid, and that the molar ratio of lactic acid formed to phosphate esterified tends to unity, i.e. per mol of phosphate esterified one mol of hydrogen passes through the pyridinenucleotide; this ratio, however, can markedly exceed unity. Needham & Pillai write "the oxido-reduction can, of course, go in the absence of adenylic acid, and the ratio would then be infinity." This observation is misleading in the light of the investigations of Warburg & Christian and Meyerhof, Schulz & Schuster. There is no disagreement, however, between the works of Meyerhof and Warburg and the experimental results of Needham & Pillai that the ratio: mols lactic acid / mols phosphate esterified may exceed a value of 2.

As pointed out by Needham & Pillai, this stoichiometric coupling between oxido-reductions and the phosphorylation of adenylic acid is closely connected with the oxido-reductive synthesis of phosphocreatine. This is clearly demonstrated by the aforesaid experiments of Meyerhof, Schulz & Schuster (27). In extracts poisoned with fluoride, one mol of phosphate combines with one mol of creatine per mol of lactic acid formed. As demonstrated by Meyerhof in experiments with "B protein" (Warburg & Christian), this reaction is exactly analogous to the fermentation equations of Harden and Warburg. In extracts to which fluoride has not been added two mols of phosphocreatine are synthesized per mol of lactic acid formed, as one mol of phosphocreatine is synthesized by means of oxido-reductive energy and the second mol of phosphocreatine by the transference of phosphate from phosphopyruvic acid. As pointed out by Meyerhof, Schulz & Schuster, as well as by Needham & Pillai, these observations make it possible to explain in chemical terms the ratio: two mols of phosphocreatine resynthesized to one mol of lactic acid formed, observed by Lundsgaard (39) during the anaërobic resynthesis of phosphocreatine following a tetanic contraction.

Very important to our understanding of the mutual relationships between the chemical changes observed in the anaërobically working muscle is Lehmann & Needham's (33) demonstration of phosphocreatine and phosphopyruvic acid as competing phosphate donators in the phosphorylation of the adeninenucleotide, and of creatine and glycogen as competing acceptors in the dephosphorylation of the adeninenucleotide. The competition between phosphocreatine and phosphopyruvic acid as phosphate donators probably explains why the breakdown of phosphocreatine per unit of work is higher in the

first part of a series of anaërobic twitches than later on. In the beginning of a series of contractions appreciable amounts of phosphopyruvate have not yet been formed, and phosphocreatine therefore is the only phosphate donor. As more and more phosphopyruvate is formed, this compound takes over the part of phosphate donor and the breakdown of phosphocreatine decreases or even stops. Undoubtedly the adenosinenucleotide is kept on the same level of phosphorylation during anaërobic work as in rest. A question which therefore may be raised is why the content of phosphocreatine does not increase again during the later part of a series of contractions when, thanks to the intermediate glycolytic reactions, the adenosine-nucleotide is kept phosphorylated, and considerable amounts of creatine, which may act as a phosphate acceptor, have been set free. This is explained by the competition between creatine and glycogen as phosphate acceptors. In this competition the glycogen dominates, favoured by the decreased pH (phosphoglyceric acid).

Our present knowledge of the chemistry of muscle extracts, based to a large extent on the fundamental observations of Lohmann, makes it possible to explain most of the well-known features in the chemistry of anaërobic muscular contraction. Most difficult to explain in chemical terms is the constant and marked liberation of inorganic phosphate in the working muscle. Most likely the inorganic phosphate does not originate directly from phosphocreatine but from adenosinetriphosphate from which inorganic phosphate is set free at a higher rate than glycogen is esterified. In experiments with muscles poisoned with iodoacetate it is easy to demonstrate (40) that the esterification of carbohydrate cannot keep pace with the liberation of inorganic phosphate.

Probably esterification by inorganic phosphate plays a greater role in the esterification of glycogen than the direct transference of phosphate from adenylypyrophosphate. That this inorganic phosphate is liberated from adenylypyrophosphate before being esterified must, however, be of some functional significance. Actually it is a very important question how the considerable amount of energy liberated by the breakdown of adenylypyrophosphate is utilized. The detection of the Cori-ester has taught us (36) that the phosphate is attached to the glycosidic linkages, and consequently the esterification of glycogen must be thermally neutral. Nor is much energy necessary for the phosphorylation of the hexosemonophosphate. Needham & Pillai suggest that the surplus of energy is used for

maintaining the endothermic zymohexase reaction. But as this reaction can take place in inactivated extracts in the absence of adenine-nucleotide and, consequently, can utilize thermal energy, it is more likely that the energy liberated by the breakdown of adenylypyrophosphate is utilized in some other way. According to the general conception, the breakdown of adenylypyrophosphate precedes the glycolytic breakdown of carbohydrate; moreover, the first-mentioned reaction is very rapid and strongly exothermic. For these reasons it is worth while to consider the possibility that the breakdown of adenylypyrophosphate may be directly associated with the restoration of the contractile substance (myosin?). Breakdown of phosphocreatine, glycolysis, and oxidations again serves to restore the adenine-nucleotide, i.e., to keep it at a constant level of phosphorylation.

In an interesting essay Needham (41) advocates a similar view and points out that if adenylypyrophosphatase is the only phosphatase present in muscle, it is obvious that all inorganic phosphate set free in the muscle must originate from the adenosinenucleotide.

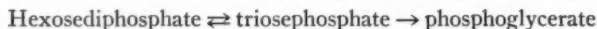
The mechanism of aërobic metabolism.—As many of the studies on the "aërobic" oxido-reduction system are made on muscle extracts, it is necessary to refer to these studies in this review.

The investigations of Szent-Györgyi and his pupils (42 to 47) on fumaric acid catalysis have been continued. Banga demonstrates that a fresh muscle extract, which has an intense respiration, by means of centrifugation can be separated into two fractions: (a) a clear supernatant fluid which contains the substrates, the coenzymes, and the dehydrogenase of triosephosphate, and (b) a sediment consisting of microscopic granuli which contains the remainder of the oxido-reduction enzymes (dehydrogenases, cytochrome-*c*, phaeohaemin). Whereas Banga studied the supernatant fluid and showed that the triosephosphate dehydrogenase precipitates in 50 per cent acetone, Straub examined the granular sediment, especially the reduction of cytochrome-*c* by hydrogen donors. Succinic acid reduced cytochrome-*c* much faster than any other substrate, while malonate inhibited the enzymic transfer of hydrogen from succinic acid to cytochrome-*c*. According to the work of Fischer & Eysenbach (48), fumaric acid, with great velocity, takes hydrogen from the hydroalloxazine enzyme ("*Leukoferment*"), which then transforms the fumaric acid into succinic acid. This fact, together with the finding of Banga that the alloxazine enzyme ("*das gelbe Ferment*") takes hydrogen from maleic acid, transforming it into oxalacetic acid,

makes it very probable that the oxalacetate-malate system comes between the pyridine and the alloxazine systems, whereas the fumarate-succinate system acts between the alloxazine and the cytochrome-*c* systems (Szent-Györgyi). This interpretation is also in accord with the thermodynamic facts (49). Szent-Györgyi regards the dicarboxylic acids as the prosthetic groups of their respective apoenzymes (malic dehydrogenase, succinic dehydrogenase).

As shown by Parnas & Szankowski (50) and by Needham & Pillai (37), the oxalacetic acid reacts with the triosephosphoric acid in the same manner as does pyruvic acid. Both reactions are able to bring about phosphorylation of adenylic acid.

Innes (51) studied the aerobic phosphorylation of creatine in muscle extracts poisoned with iodoacetate. He finds that the aerobic formation of phosphocreatine in the presence of hexosediphosphate and iodoacetate is accomplished by a breakdown of phosphopyruvate. The phosphopyruvate is regenerated by the following reactions:

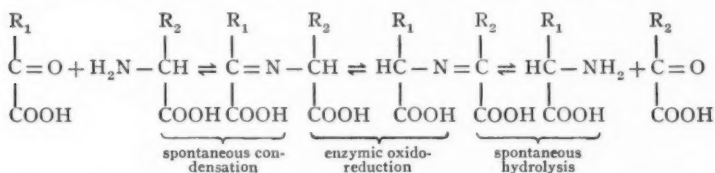


Whereas the dismutation of the triosephosphate is inhibited by iodoacetate, the oxidation is not. A coupling of the oxidation of triosephosphate with phosphorylation of creatine by inorganic phosphate does not occur in the presence of iodoacetate, but is possible in fluoride-poisoned extracts. The mechanism of the aerobic phosphorylation of creatine is apparently almost the same as the anaerobic, especially if the oxidation takes place through the Szent-Györgyi system. As appears from the investigations of Needham & Pillai (37), not every oxido-reduction is able to phosphorylate adenylic acid.

Krebs (52) has a quite different conception of the dicarboxylic acid systems. He finds that even very small amounts of added citric acid increase the respiration of muscle tissue considerably. As the citric acid does not disappear this effect must be catalytic. In the presence of arsenite, citric acid is transformed and accumulated as α -ketoglutaric acid (oxidative decarboxylation). Krebs finds further that a considerable formation of citric acid takes place if large amounts of oxalacetic acid are added to muscle tissue. Starting from these findings, and using Knoop & Martius' (53) scheme for the oxidative decarboxylation of citric acid, Krebs presents the hypothesis that oxalacetic acid acts as a carrier of a metabolic product of

sugar (acetic acid?). When acetic acid is condensed with oxalacetic acid, citric acid alone is formed. This condensation is then succeeded by several oxidations in which two molecules of carbon dioxide are set free; the oxidations continue until the oxalacetic acid is rebuilt. This hypothesis is in reality a compromise between Szent-Györgyi's and Thunberg's interpretations of the role of the dicarboxylic acids in carbohydrate metabolism. Krebs' experiments are criticised by Breusch (54) who holds the opinion that the formation of citric acid from oxalacetic acid is partly non-enzymic.

In two very interesting papers by Braunstein & Kritsman (55) a new and significant reaction, the so-called "*Umaminierung*," is described. If *l*(+)-glutamic acid is added to minced muscle tissue the glutamic acid is converted into α -ketoglutaric acid (oxidative deamination); the ammonia however is not liberated but is transferred to pyruvic acid, which is then converted into *l*(+)-alanine (reductive amination). The reaction is reversible and takes place with considerable velocity. The scheme of the reaction is as follows:



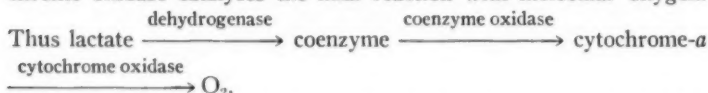
The intermolecular transfer of ammonia apparently takes place only if either the donator (amino acid) or the acceptor (α -keto acid) is a dicarboxylic acid. If, however, small amounts of an aminodicarboxylic acid or an α -ketodicarboxylic acid is present the transfer of ammonia between two monocarboxylic acids is started. The dicarboxylic acids act as carriers in the transfer of amino groups. The analogy to the system of Szent-Györgyi is striking.

Quite recently Dewan & Green (56) have described a very simple oxidation system which may be of great interest: If a phosphate extract of washed and ground muscle tissue is added to a mixture of reduced coenzyme I and one of its special proteins, the rate of oxidation of reduced coenzyme is increased some twentyfold. The factor which catalyses the oxidation of reduced coenzyme I can be sedimented by centrifuging and washed by repeated suspension in water without any loss of activity.

According to Dewan & Green, every coenzyme-dehydrogenase

system known in animal tissues can be resolved into two catalytic components: (a) the water-soluble dehydrogenase which catalyses the oxidation of the substrate by the coenzyme (coenzyme reductase); (b) the insoluble complex factor which catalyses the oxidation of reduced coenzyme. These investigators¹ have succeeded in resolving this complex factor into three insoluble components: (a) the coenzyme factor (coenzyme oxidase); (b) cytochrome-*a* and -*b*; (c) cytochrome oxidase.

The coenzyme oxidase catalyses the first oxidation and the cytochrome oxidase catalyses the final reaction with molecular oxygen.



The coenzyme oxidase is not identical with flavoprotein, which shows practically no activity as a factor in this system.

Green, Needham & Dewan (57) have published some very interesting investigations on the dehydrogenase of triosephosphate. This enzyme catalyses the anaërobic as well as the aërobic dehydrogenation of triosephosphate.

The enzyme system which catalyses the anaërobic oxido-reduction: triosephosphate—coenzyme—pyruvic acid, is termed mutase. According to Green and coworkers, each mutase consists of a protein with two active groups, one of which activates the substrate and catalyses the oxidation of the substrate by the coenzyme; the other activates the α -ketonic acid and catalyses the reduction of the α -ketonic acid by the reduced coenzyme. The coenzyme is the liaison agent between the two active groups.

The mutase system cannot react with oxygen carriers such as flavin in the absence of a thermolabile factor found in skeletal and cardiac muscle extracts. The factor is non-dialysable and is sedimented by high-speed centrifugation. It is not identical with any of the known carriers. The factor is present in the usual preparations of dehydrogenases.

Green, Needham & Dewan, moreover, have carried out experiments (57) which, on the basis of the Embden-Meyerhof scheme of glycolysis and the discoveries of Meyerhof & Lohmann (58) of the aldolase reactions, throw considerable light on the path of carbo-

¹ The nomenclature of Dewan & Green is not quite clear and has been slightly changed in this present review.

hydrate synthesis from lactate. As we know, triosephosphate and pyruvic acid in the presence of the appropriate mutase and coenzyme I react until all of the triosephosphate is oxidised. That is to say, equilibrium is reached only when the reaction has proceeded practically to completion. In order to reverse the forward reaction, some means must be found of shifting the equilibrium point. Since cyanide combines with both triosephosphate and pyruvate, the use of this reagent should enable the reaction to proceed in the reverse direction, that is to say: lactate + phosphoglycerate \rightarrow pyruvate + triosephosphate. The lactate acts as reductant, the phosphoglycerate as oxidant. The velocity of the reaction, as measured by the disappearance of acid groups, is rather high—about 350 c.c. carbon dioxide in ten minutes. Only *l*(+)-lactate and *l*(-)-malate are active as reductants for phosphoglycerate. In addition to the production of alkali-labile phosphate, a considerable liberation of inorganic phosphate is observed coincident with the oxido-reduction.

Green, Needham & Dewan are of the opinion that the physiological synthesis of carbohydrate from lactate takes place in the following manner: Given a trace of phosphoglycerate, reaction with lactate will produce triosephosphate and pyruvate; this reaction will proceed to an appreciable degree if the triosephosphate formed is continually removed, as would happen if, for example, the hexosediphosphate into which it would be converted were being transformed into glycogen; as for the pyruvic acid formed in the oxido-reduction, once it has been phosphorylated, its conversion into a further supply of phosphoglycerate to react with lactate will readily follow. Thus, given an energy source for the phosphorylation of pyruvate and for other energy-requiring reactions, it should be possible to convert lactate quantitatively into glycogen.

As pointed out to the author of this review by H. Kalckar in a discussion on the above-mentioned paper, the principle of sugar formation in animal tissue may be described as a liberation of hydrogen from the hydrogenated oxidant of glycolysis (hydrogenated pyruvic acid, i.e., lactic acid) and a transfer of this hydrogen to a sugar acid (phosphoglyceric acid). The formation of sugar in animal tissue reflects on a small scale the sugar formation of the green plants. In the vegetable kingdom the energy of light makes possible the liberation of hydrogen from the hydrogenated oxidant of respiration (hydrogenated oxygen, i.e., water), this hydrogen being transferred to the carbon dioxide.

The anaërobic metabolism of muscle.—Only a very few papers dealing with the anaërobic chemical changes in the intact muscle have been published this year.

Cattell & Feit (59) draw attention to the fact that the efficiency (T/H) decreases when an isolated frog muscle is kept under anaërobic conditions for some hours. If the muscle is stimulated for short intervals during anaërobiosis, this decrease in T/H does not occur or is much less pronounced than in resting muscles. As the decrease in efficiency is probably due to chemical changes in the muscle, this result seems to indicate some difference between the nature of the anaërobic chemical processes in the resting and the active muscle.

Deuticke & Ebbecke (60) have demonstrated that in frog muscles "stimulated" by exposure to high pressures (300 to 1500 atms.) the same chemical changes occur as in muscles stimulated electrically under anaërobic conditions. The breakdown of phosphocreatine precedes the lactic acid formation as in a series of anaërobic twitches. Special interest may be attached to the statement that after exposure of short duration (less than 8 seconds) to moderately high pressures (500 to 800 atms.) an increase of the adenosinetriphosphate is demonstrable. This observation corresponds to that made some years ago by Embden and coworkers (61), and later criticised by Lohmann (62), that an increase in adenosinetriphosphate is demonstrable in muscles stimulated by immersing in liquid air. This observation has again raised the question of whether adenylic acid (or adenosinediphosphate) is a normal constituent of the resting muscle.

The effect of acetylcholine on the anaërobic metabolism of the intact frog muscle has been studied by Nachmansohn (63). Moderate concentrations of acetylcholine provoking a marked contracture have no effect on the anaërobic metabolism. Large concentrations (100 to 200 $\mu\text{g. per c.c.}$) increase the breakdown of phosphocreatine and the formation of lactic acid. This increased metabolism continues for hours though the contracture provoked is only of short duration. The effect of acetylcholine on phosphocreatine is contrary to that provoked by adrenaline but corresponds to the effects of other parasympathomimetic factors (pilocarpine and potassium ion).

The aërobic metabolism of muscle.—A problem still open to discussion is that of the extent to which the well-known anaërobic metabolic processes participate in the metabolism of the aërobic working muscle, i.e., muscles working with unimpaired circulation and adequate oxygen supply. Most authors dealing with this problem

seem to favour the assumption that lactic acid formation plays no role, or at any rate only a minor one, in the metabolism of aerobically working muscles. The papers which must be taken into consideration in judging this problem may be divided into groups which deal with the following: (a) Factors controlling the catalysis of glycolysis; (b) the nature of the fuel utilized during aerobic work; (c) the chemical changes in aerobic working muscles; (d) fluctuations in blood lactate during exercise; (e) the time relations of oxygen consumption in active muscle.

(a) Gemmill & Hellerman (64) have demonstrated that certain organic mercury compounds and iodine inhibit glycolysis in extracts of frog muscle and that this inhibition may be abolished by the use of cysteine or glutathione. In the case of iodine, ascorbic acid also abolishes the inhibition. The authors suggest that the inhibition is due to reversible chemical action upon substituent sulphhydryl groups of the enzyme molecules. In the case of iodine inhibition, it is probably a question of an oxidation of sulphhydryl groups. These results, therefore, correspond closely to those obtained by Lipmann in 1933 (65). The authors are disposed to interpret their results as an oxido-reductive control of the catalysis of the glycolytic process in muscle. This interpretation is consistent with that given by Lipmann. Though the interpretation is not certain, the results are suggestive of a depression or even abolishment of glycolysis in muscle under aerobic conditions.

Hahn and coworkers (66) have demonstrated the existence in muscle tissue of a factor which controls aerobic glycolysis. If muscle pulp is shaken with phosphate buffer and the washed muscle pulp and the extract are separated, the glycolysis in both fractions is independent of the presence of oxygen. If, however, part of the extract is added to the washed muscle pulp the glycolysis of this recedes under aerobic conditions. Obviously a factor is extracted which is able to re-establish the aerobic recession of glycolysis (Pasteur effect) in washed muscle pulp. Further experiments (67) have shown that the active factor is not precipitated in 50 per cent acetone. It is stable to boiling and can be precipitated by barium acetate. The barium precipitate can be stored for a considerable time without losing its activity. Judging from these observations the active factor may be a nucleotide. The mechanism of this aerobic inhibition of glycolysis is so far unknown.

The postulated constancy of the oxidative quotient has been con-

sidered a kind of proof of the correctness of the assumption (Meyerhof) that the recession of lactic acid formation during aërobiosis (Pasteur reaction) is due to a constant resynthesis of the lactic acid actually formed into glycogen. The demonstration by Cori, Cori & Hegnauer (68) that considerable amounts of hexosemonophosphate, along with lactic acid, are rebuilt into glycogen in frog muscle during aërobic restoration after short tetanic stimulation, shows that the original determination of the oxidative quotient by Meyerhof (69) cannot be considered reliable. The oxidative quotient for the disappearance of lactic acid may be of any order of magnitude and quite different from the value calculated by Meyerhof from the ratio: lactic acid disappeared / glycogen resynthesized.

(b) As there are good grounds for supposing that an extensive formation of lactic acid during aërobic work would lead to an exclusive oxidation of carbohydrate, all experimental evidence of a combustion of materials other than carbohydrate in the working muscle may be considered as circumstantial evidence in favour of the assumption that no lactic acid formation takes place in aërobically working muscles. The inability of muscle to utilize certain "foodstuffs" as a fuel during work may be considered, on the other hand, circumstantial evidence in favour of the assumption that the oxidative metabolism of the working muscle is a specific (probably pure carbohydrate) metabolism.

A dependence of the respiratory quotient on lactic acid formation may be the cause of the increase in R.Q. of isolated frog muscles from the normal average of 0.89 to an average of 1.0, observed by Saslow (70) when muscles were treated with subcontracture concentrations of caffeine. In caffeinized frog muscles the anaërobic lactic acid formation was increased to 80 to 90 mg. per cent per hour.

Experiments on the isolated frog heart (71) and on the isolated mammalian heart poisoned with iodoacetate (72) indicate that under such conditions the heart resorts to fat oxidation for supply of necessary energy. A frog ventricle poisoned with iodoacetate and perfused with Ringer solution in air until the available metabolites are exhausted can be revived by the addition to the perfusion fluid of fatty acids (propionic acid to decolic acid).

In the aglycaemic mammalian heart, working apparently successfully with an R.Q. of 0.7, there is, however, a loss of phosphocreatine and pyrophosphate. In the mammalian heart poisoned with iodoacetate there is also a loss of phosphocreatine and a remarkably

pronounced loss of pyrophosphate, indicating that the metabolism is not completely unimpaired.

The fact that the rate of oxidation of alcohol does not increase during muscular exercise is explained by Lundsgaard (73) as due to the inability of muscle tissue to oxidize alcohol directly. Only after a preliminary partial oxidation to acetic acid in the liver is alcohol utilized in the tissues. The primary oxidation in the liver, which may be supposed to be unaffected by an increased metabolism in the striated muscles, is therefore the limiting factor for the rate of disappearance of alcohol from the system. This conception is based on experiments on alcohol oxidation in artificially perfused isolated livers and hind limb preparations.

(c) Sacks, Sacks & Shaw (74) have studied the chemical changes in the gastrocnemius muscle of the cat with intact circulation stimulated artificially by single shocks. Two different rates of stimulation were employed: one shock per second and two shocks per second. As "the initial contractions developed a definitely higher tension than the subsequent ones, but before the end of the second minute the tension per twitch had reached a practically constant level," the authors conclude that "a steady state" is arrived at within two minutes. Groups of experiments with different periods of stimulation (one minute, two minutes, five minutes, and ten minutes) were carried out. The outcome of the experiments may be summarized as follows. At one twitch per second the lactic acid concentration in the muscle increases rapidly at first and then more slowly. Within five to ten minutes there is even a marked decrease in lactic acid concentration. The phosphocreatine content decreases rapidly at first and then more slowly. In between five and ten minutes there is a definite increase in the phosphocreatine content. At two twitches per second the lactic acid concentration reaches a maximum within one to two minutes and then remains constant. The phosphocreatine concentration decreases rapidly during the first two minutes and then remains fairly constant or increases a little. The authors still calculate the breakdown of phosphocreatine without taking the formation of hexosephosphate into consideration. They conclude that during a "steady state" a moderate formation of lactic acid is going on. In the experiments with a rate of two twitches per second the diffusion of lactic acid to the blood and oxidation of lactic acid counterbalance the formation. In the experiments with a rate of one twitch per second the removal of lactic acid is faster than its formation. "This

continuous formation may be due to local areas of oxygen deficiency, in which contraction is accomplished by anaërobic reactions, or it may be another example of the aërobic glycolysis seen in other tissues with a high metabolic rate." A steady state in the sense of the authors may of course be obtained even if the muscle is working partly anaërobically. The fact that at the lower rate of stimulation the lactic acid formation apparently decreases after the first five minutes suggests that under conditions more favourable to the oxygen supply than in an artificially stimulated muscle the lactic acid formation may even stop completely. The authors still favour the conception of the phosphocreatine being a "buffer substance."

(d) Using the same general arrangement as in the experiments just mentioned, Sacks & Sacks (75) have compared the lactate concentration of arterial and venous blood, one to three minutes after the commencement of stimulation, with the concentration of lactic acid in muscle. The venous blood sample was drawn from the popliteal vein, all branches of which, except those emerging from the gastrocnemius, having been tied off. The tibial and peroneal nerves were cut just distally to this muscle. The outcome of the experiments, that lactic acid concentration in the venous blood is much lower than the concentration in the muscle, may be considered as a demonstration of the rather slow diffusion of lactic acid from the muscle to the blood. However, the experiments can hardly be considered as proof that an equilibrium between the working muscles and the blood cannot be obtained during exercise, and for the following reasons: (i) the samples were drawn at a point of time when the lactic acid concentration in the muscle was increasing; (ii) owing to the very small mass of muscle stimulated the lactate concentration in the arterial blood did not increase; (iii) the conditions for diffusion of lactic acid may be much less favourable than in a muscle working under normal conditions.

Newman, Dill, Edwards & Webster (76) have made determinations of the rate of lactic acid removal in exercise. The blood-lactate concentration was traced in subjects who previously had run to exhaustion in one minute. In some experiments the short strenuous work was followed by complete rest, in others by moderate work on a treadmill for forty-five minutes at rates up to twelve times the basal rate of oxygen consumption. The moderate work in itself did not cause any increase in blood-lactate concentration. The rate of removal of lactic acid during exercise increased approximately proportionately with the metabolic rate up to some critical level of

activity, different for each subject and corresponding to the level of activity which in itself would provoke an increase in the blood lactate of the individual. According to these results a decrease in the concentration of blood lactate during exercise of the same rate and even of a somewhat higher rate than that observed after the exercise has stopped must be considered evidence of a formation of lactic acid during work. Only in cases in which the blood lactate does not increase or reaches the basal level during the exercise, is the assumption tenable that no lactic acid is formed. The fact that the rate of removal of lactic acid is proportional to the metabolic rate up to the level of activity which in itself provokes an increase in the blood lactate seems to favour the assumption that no lactic acid is formed when no lactic acid appears in the blood.

Johnson & Edwards (77) have determined the lactate and pyruvate in blood and urine after exercise. The demonstration of an increase in pyruvate in blood after exercise may be said to support the Embden-Meyerhof scheme for muscle glycolysis *in vivo*. The pyruvic acid 2,4-dinitrophenylhydrazone was isolated from blood and urine and identified. The increase in pyruvate in blood amounted only to 2 to 4 per cent of the increase in lactate.

(e) Tracing of the time relations of oxygen consumption in active muscle has been made possible by the ingenious technique developed by Millikan. Though the details of Millikan's work were not published until this year (78), the technique and main results were reported on rather fully in the last volume of this *Review*.² By tracing the reduction of myoglobin in the soleus muscle of the cat during rest and tetanic stimulation it was shown that when contraction takes place the oxygen consumption starts to increase in less than 0.2 second (the response lag of the instrument) and reaches its maximum inside of one second, if not sooner. This valuable information makes the assumption possible that oxidations may take over the energy production at a very early stage in the chain of energy-yielding reactions known to occur in the anaerobically working muscle; at what stage, however, it is thus far impossible to say. The validity of the calculations of the absolute rate of oxygen consumption and the comparison with the rate of oxygen consumption in muscles during severe muscular exercise are dubious. The energy liberation in a muscle during artificial tetanic contraction cannot be directly compared with the energy output in muscles during exercise. In the artificially stimulated muscle the motor units are working

² *Ann. Rev. Biochem.*, **6**, 409 (1937).

synchronously and remain contracted during a maximal tetanus. The energy output is constant. During exercise the energy output in the muscles is intermittent and even during the contractions the energy output of the individual motor unit is probably intermittent and the maximal rate of energy output consequently much higher than the average energy output of the total bulk of muscles as judged from the oxygen consumption of the individual. In view of the intermittent activity of the individual motor unit the function of the myoglobin as a short-time oxygen store that may tide the muscle cells over from one contraction to the next is probably of the greatest significance.

Miscellaneous.—Acetylcholine esterase in muscle tissue was determined by Nachmansohn (79). From the first series of experiments Nachmansohn concluded that the concentration of esterase is far too low to explain the rapid hydrolysis of acetylcholine postulated in the theory of acetylcholine as the chemical transmitter of the nerve impulse. However, further experiments (80) have removed this difficulty. A comparison between the esterase concentration in the upper third of sartorius muscles containing no nerve endings and the middle third containing numerous nerve endings revealed a definitely higher concentration in the latter than in the former. From this difference Nachmansohn has calculated the esterase concentration in the end plates to be 10,000 to 20,000 times the concentration in muscle tissue.

Soskin, Essex, Herrick & Mann (81) have studied the influence of epinephrine on the utilization of glucose in the skeletal muscles. The arterio-venous glucose deficit was determined during alimentary and epinephrine hyperglycaemia of equal magnitude. No difference between the two series of experiments could be demonstrated. Jacob & Mond (82) point out that the utilization of glucose in an artificially perfused muscle is dependent on the concentration of electrolytes in the perfusion fluid. Perfused with pure glucose solution the muscle loses its permeability to glucose.

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LIVER AND BILE*

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Akerren (1) has published a monograph dealing with experimental changes in liver function, and Greene *et al.* (2) have reviewed the clinical aspects of liver and biliary tract function. It is interesting to note that while in the past the literature on the liver has been much concerned with carbohydrate metabolism, interest in the past two years has shifted more to the metabolism of the lipids. As the chemistry of the bile acids has been elucidated, there has also been an increase in the number of investigations concerned with various phases of biliary secretion. Other short reviews of the literature on hepatic function may be mentioned (3, 4, 5, 6).

CARBOHYDRATE METABOLISM

Bell (7), continuing his studies on the chemical constitution of glycogen, confirms the fact that it ordinarily consists of 12 glucose units, and finds that when galactose is fed the glycogen formed is made up of 18 glucose molecules. These two glycogens show no significant differences in their properties. Guelin-Schedrina (8) has found that glycogen appears in the chick-embryo liver on the eighth day of incubation, while the islets of Langerhans differentiate on the ninth day; the injection of insulin into five- and six-day embryos did not cause glycogen to be formed. Ascorbic acid given to guinea pigs over a twelve-day period can increase the liver glycogen 60 per cent, according to Hirsch (9), and can even offset the glycogenolytic effect of thyroid extract. Lundsgaard *et al.* (10) report that the isolated cat liver does not utilize glucose from perfusion fluid even when insulin is added, although fructose and lactic acid are utilized and stored as glycogen. Pancreatectomy forty-eight hours prior to the experiment had no effect. In perfused dog liver Fiessinger and his collaborators (11, 12) found that glycogen decreased and the glucose content of the blood used for perfusion rose. When insulin was added the glucose output was above normal in three cases and unchanged in three others. Kepinov, on the contrary, using guinea pig livers in perfusion experiments, found that insulin inhibits glyco-

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genolysis (13). He also reported (14, 15) that when perfusion is continued for some hours the liver loses a substance that renders it capable of responding to epinephrine and that the response can be restored by adding liver, muscle, or hypophyseal extract to the perfusion fluid. Liver extract from hypophysectomized animals, however, has no effect and he postulates that epinephrine glycogenolysis requires the presence of an additional hormone from the pituitary gland.

Tsai & Yi (16) have studied the sugar content of hepatic inflowing and outflowing blood in unanesthetized, angiotomized cats, and state that the failure of the liver in the decapitate cat to retain glucose when this sugar is absorbed from the gut is due to some interfering factor in the experimental procedure. Cherry & Crandall (17), from similar experiments on angiotomized dogs, also conclude that the liver retains glucose when it is given by mouth, even when the blood-sugar level has ceased to rise. Since these findings are contrary to those of other investigators who used anesthetized or decapitate animals,¹ it appears that much judgment must be exercised in drawing conclusions from investigations on such preparations. Cherry and Crandall further note that in the fasting dog, the liver gives up 2 to 22 (average 8) mg. of glucose per 100 cc. of blood, and that any retention of lactic acid by this organ tends to cease (lactic acid may even be liberated) during glucose absorption. De Lucia & Cedrangolo (18) find no glycogen storage after galactose administration to fed rats, although storage occurs if the rats are starved for 24 to 28 hours; glucose caused storage in the fed animals.

Reid (19) observes no correlation between the specific dynamic action of an amino acid and its ability to form glycogen. Under chloralose anesthesia glycogen was laid down during infusion (cats) with glucose, lactic acid, glycerol, and alanine but not with propionic, glutamic, or aspartic acids. In a later study (20) he found that the deposition of glucose is not increased when insulin is given; removal of the adrenal medulla does not alter the results. Bach & Holmes (21) state that liver slices from starved rats form fermentable reducing substance from lactate, pyruvate, alanine, aspartic acid, glutamic acid, and arginine; glycine may slightly decrease the amount of reducing substance present. Insulin causes a marked decrease in the formation of reducing substance from amino acids and inhibits urea formation as well, but does not affect carbohydrate synthesis

¹ Cf. *Ann. Rev. Biochem.*, **5**, 431 (1935).

from lactate or pyruvate; its action therefore appears to be on the deaminization mechanism. Cross & Holmes (22), using the same technique, demonstrate glycogen synthesis *in vitro* and find that the normal liver uses butyrate, succinate, and glycerate but not acetate as glycogen precursors. Intoxication of the animal from which the liver slice is taken with diphtheria toxin depresses carbohydrate synthesis from most compounds. In the fasting rat a depression by insulin of glycogen synthesis from methylglyoxal and from ketol has been observed by Stöhr (23, 24). Fukase (25) states that insulin accelerates glycogen formation in the livers of rabbits starved four or seven days; bile acids act similarly. Aubertin & Castagnou (26) found that the injection of insulin into dogs with ligated pancreatic ducts had the same effect as in normal animals if the livers were still normal. After fatty degeneration had occurred the action of insulin was greater and more prolonged. According to Fletcher & Waters (27), small doses of insulin which do not affect glycogen synthesis when glucose is being absorbed by rats do affect it when fructose is given; the liver-sugar blood-sugar ratio during absorption of both sugars was close to 1.1.

Chaikoff, Holtom & Reichert (28), and Corey & Britton (29), have found the liver glycogen of hypophysectomized animals maintained in good nutritional state to be within normal limits; the former authors worked with dogs, the latter with rats. The abnormalities of carbohydrate metabolism that are present after removal of the hypophysis cannot, therefore, be ascribed to deficient glycogen storage. Samuels, Schott & Ball (30) studied further the delayed removal of glucose from the blood stream of hypophysectomized animals and concluded that it is due to decreased glycogenesis, presumably in the liver, and that this effect cannot be mediated (at least entirely) through the adrenals. Bachman & Toby (31) found that the hyperglycemic response of hypophysectomized rabbits to epinephrine is highly variable. It may be good if the liver glycogen is high, but is always poor if the animals have been fasted. Buell, Anderson & Strauss (32) report that adrenalectomized rats fail to convert *D*-lactic acid into liver glycogen at a normal rate, even when kept in good condition on a high salt diet; when given cortical extract normal conditions are restored. The glycogen stores of fasted adrenalectomized rats are low. Hochfeld (33) indicates that cortical hormone is capable of increasing the liver glycogen of normal animals. He finds a higher glycogen in rats fed with fat than in those on a mixed diet if the

determination is made following one day of fast, and a still higher glycogen if cortical hormone is given. He believes that this indicates the formation of carbohydrate from fat under the influence of the hormone. Matsuoka (34) states that cortical hormone can promote liver-glycogen formation even in rabbits poisoned with chloroform, and that the same is true of thyroxin, although previous investigators have almost uniformly reported that thyroxin is glycogenolytic.

Schulze (35) finds that subacute carbon monoxide poisoning in white mice decreases liver glycogen. The decrease is temporary with four days of poisoning and is prolonged when poisoning is continued 12 to 26 days. Inagaki (36) notes various disturbances of hepatic carbohydrate metabolism from the administration of sodium phenolate, benzoic acid, etc. Yokoyama (37) reports that yohimbine will inhibit the mobilization of glycogen produced by stimulation of the splanchnics. According to Lundsgaard (38), phlorhizin does not affect hepatic carbohydrate metabolism in doses which inhibit reabsorption in the kidney tubules.

LIPID METABOLISM

Best & Channon (39) have presented a complete discussion of the results obtained in their studies on liver lipids, and the effects of choline and other substances upon them. They conclude that qualitatively the findings of their two laboratories are the same, the differences being only in the degree of effect obtained. The dietary regime necessary to observe the effects of choline is given in detail, as are conclusions relative to the action of choline on fatty livers produced by high fat or high cholesterol diets. They agree that the protein of the diet also controls the lipid content of the liver. Best, Grant & Ridout (40) find that while casein has a definite choline-like action, gelatin exerts little if any effect; the actions of casein and choline are qualitatively indistinguishable. A further discussion of the effects of cholesterol and choline on liver fat is presented by Best & Ridout (41), who note that when the rat receives small amounts of cholesterol the addition of choline to the diet produces a fall in both glycerides and cholesterol esters. With larger amounts of cholesterol the first effect of choline is on the neutral fat which may fall while the cholesterol fraction is increasing. Maclean, Ridout & Best (42) state that while fat deposition on low choline diets may be irregular over short periods of time it becomes more uniform when the experiment is prolonged. Animals on a minimal amount of choline lose weight, and store little fat in the usual fat depots. When choline is given the weight gains are nor-

mal, as is fat storage. Liver-function tests may show functional impairment when the diet is low in choline, and the ketone excretion tends to be higher. They conclude that choline favors the normal distribution of body fat and prevents failure of liver function. Channon, Platt & Smith (43) have found homocholine more effective than choline in the prevention of fatty liver. Tripropyl- β -trihydroxyethylammonium hydroxide is without effect, although as previously shown (44) the triethyl compound is nearly as active as choline. In other communications by Channon and his collaborators (45, 46, 47, 48) they find that caseinogen seems to differ from choline in its lipotropic effect, since it permits an increase in the absolute amount of phosphatide. By using various protein percentages in a diet containing 20 per cent of fat and 2 per cent of choline it was possible to produce in rats fatty livers of widely varying composition. The addition of 80 mg. of choline per day largely prevents the deposition of neutral fat but only partially that of cholesterol esters. Rats receiving a low choline diet containing 5 or 40 per cent of caseinogen, with and without cystine, deposit much more fat in their livers when cystine is given. The nature of the fat deposited in the liver is decidedly influenced by the type of fat fed. Finally, these investigators observe that a hydrocarbon, squalene, given as 1 per cent of the diet causes an increase of 50 per cent in liver sterol and 33 per cent in fecal sterol without change in carcass sterol. They give reasons for believing that squalene is not converted into sterol in the body. Tucker & Eckstein (49) confirm Beeston & Channon (46) relative to the effect of cystine in increasing liver lipid. They further find that 0.5 per cent of methionine in the diet greatly decreases the liver lipid even when the diet is such as to produce fat deposition, and they believe that this may explain the observations of Channon and Wilkinson² (1935) on the effect of casein. Kaplan & Chaikoff (50) continue their studies on liver lipids in dogs; they are almost the only investigators to use large animals. They find that the effect of choline is slow even with large doses, requiring three to fifteen weeks. Raw pancreas acts similarly, but according to their observations, choline is not the factor in raw pancreas that produces a rise in blood lipids. Deuel *et al.* (51) observe in rats less ketonuria in the first two days of fasting and more on the next three days when choline is given with a high butterfat diet. Choline given to controls during fasting decreased ketosis. They conclude that choline does not increase the rate of fat oxidation

² *Ann. Rev. Biochem.*, **5**, 437 (1936).

and that it prevents deposition in the liver but not elsewhere in the body. According to Mann & Quastel (52), choline increases the respiratory rate of rat-liver slices (see Quastel, 1935)³ and there is a disappearance of choline with a production of betaine aldehyde; guinea pig liver and kidney are inactive.

Dragstedt and his collaborators (53) conclude that the fatty degeneration and infiltration of the liver which occur in depancreatized dogs are not due to the absence of pancreatic juice from the intestine. In a second communication (54) they state that the amount of choline contained in that quantity of raw pancreas which is adequate for prevention of fatty livers in depancreatized dogs, will not of itself prevent such changes. They conclude that a fat-metabolizing hormone which they term "lipocaic" must be present and that it is active by mouth. MacKay (55), using rats, confirms the fact that an extract prepared by the method of Dragstedt *et al.* will prevent fatty livers. Chaikoff & Kaplan (56) question these findings, since they observe that the deposition of fatty acid may be very uneven; in the same animal one lobe of the liver may contain 4.3 per cent and another 19.7 per cent. The determinations of Dragstedt *et al.* were made on samples taken at biopsy. Kaplan & Chaikoff further note (57) that while some depancreatized dogs may develop fatty livers after 3.5 weeks, other animals may show only 6.6 per cent fat after 14.5 weeks. After 16 weeks the livers were consistently fatty, although spontaneous regression may occur on long survival. They point out that the experiments of Dragstedt *et al.* were carried out over periods of three to six weeks. They also find the lipotropic factor of raw pancreas to be heat stable, and believe that the amount of raw pancreas needed for prevention of fatty livers is greater than that stated by Dragstedt *et al.*

Dietary factors other than choline which influence hepatic fat deposition have been studied by several investigators. Eckstein & Treadwell (58) report that the sterol content of liver of growing rats is greater when much corn or soybean oil is fed, but reach no conclusion as to whether this increase is caused by an increased intake of sterol, fat, or both. Channon, Jenkins & Smith (59) fed rats with diets containing 40 per cent of various fats for fourteen days. The amount of fat deposited in the liver varied with the type fed, but there was no relationship between the amount of liver fat and the amount of carcass fat. The saturated fats in the liver re-

³ *Ann. Rev. Biochem.*, **5**, (1936).

sembled those in the carcass, while the unsaturated did not; liver phospholipids had no relation to those in the carcass. They conclude that if desaturation of the lower fatty acids occurs in the liver the desaturated fats are not stored. Rubin, Present & Ralli (60) analyzed the liver lipids of 33 normal dogs that had received up to 30 gm. of various fats with and without 10 gm. of crude lecithin and found no statistically significant differences. Okey & Yokela (61) found that rats fed with egg yolk to furnish 1 per cent cholesterol developed fatty livers in spite of the 2.3 per cent phospholipid content of the diet. When the egg-yolk protein and cholesterol were fed with hydrogenated cottonseed oil (no lecithin), liver fats were lower than on egg yolk. Raw or dried liver was fed by Beeston & Wilkinson (62), who found that it produced fatty livers, confirming earlier studies. Added choline was not preventative; the amount already present in the diet may have exerted a maximal effect. Flock & Bollman (63) observed that the neutral fats of the plasma in dogs are not elevated above the normal range during the production of fatty liver by high fat diets or by such a diet plus alcohol.

Ketogenesis in rabbit-liver pulp has been studied by Leites & Odinov (64), who report that the production of acetoacetic and β -hydroxybutyric acid is more pronounced when animals have been fasted 48 hours. The optimum pH was 5.6 for acetoacetic acid production and 7.0 for β -hydroxybutyric in non-fasting animals, but was 5.6 for both when the animals had fasted. The lower the preformed ketone-body content, the greater the amount produced. Butyric acid stimulated intensive ketogenesis, but sodium acetate and pyruvic acid were without effect. Mirsky (65) found that the ketogenic principle of the anterior lobe of the hypophysis is inactive in eviscerated animals, and later (66) noted that both insulin and ergotamine will counteract the ketogenic effect of this extract. He suggests that all of these agents act upon the liver, and that insulin and ergotamine owe their antiketogenic effect to their ability to inhibit glycogenolysis. Deuel, Hallman & Murray (67) report that rats fasted five days show no ketosis when the diet has been low in fat, while a ketosis occurs if the fast follows a high fat diet. There is, however, no definite relationship between the ketosis and the percentage of fat in the liver. Females exhibit more ketonuria than males, but have no higher liver fat. The authors suggest that, perhaps because of a difference in the character of the fats, those in the livers of females disappear more rapidly on fasting.

Anselmino, Effikeman & Hoffman (68) injected hypophyseal extracts containing the "carbohydrate metabolism hormone" and found a decrease in the unsaturated fatty acids of the liver which reached a maximum in six to eight hours. A decrease in liver glycogen was also noted, although blood ketone bodies were not changed. When the same authors (69) injected rats and cocks with the "fat metabolism hormone," the unsaturated fatty acids increased and reached a maximum in six hours, but the effect on total fatty acids was variable. After fat feeding a substance having the effects of the "fat metabolism hormone" appeared in the blood. Chaikoff and his collaborators (70) have studied the effect of hypophysectomy and of hypophysectomy plus pancreatectomy. They find that absence of the pituitary gland does not alter the lipid content of the liver in dogs, nor does it inhibit the accumulation of lipids in the liver after pancreatectomy. In 5 of 16 hypophysectomized dogs the blood lipids were elevated, but were normal in the remaining animals. The hypophysectomized-pancreatectomized animals were capable of storing liver glycogen, and some exhibited ketosis. The importance of the nutritional state for the metabolism of the animals is emphasized. Best & Campbell (71) produced intensive fatty infiltration of the livers of fasting rats by injections of an anterior lobe fraction for three days. There was also a decrease in total body fat and an increased excretion of ketone bodies. Similar but less marked effects were produced in fed rats. Posterior lobe extracts produced only a slight rise in liver fat. MacKay & Barnes (72) confirm the fact that ketogenic anterior-pituitary extracts cause fat deposition in the liver, and report that in rats adrenalectomy prevents the ketosis and fat deposition caused by the extract as well as reducing the amount of fat deposited in the liver during fasting. MacKay (73) further finds that adrenalectomy decreases the fat deposition in the liver and the amount of ketosis produced by fat administration, alkalosis, or epinephrine. Adrenalectomized rats that have been fed a high fat diet lose fat from their livers more rapidly during fasting than do controls on the same diet.

Flock, Bollman & Mann (74, 75) have studied the effect of diet and other factors on the phosphate compounds in the liver of the dog. Ordinary variations in diet produce but little change. When a high fat diet is continued long enough to produce extremely fatty livers the total phosphate decreases, the major part of the decrease being in the acid soluble fraction with but little change in phospholipid. Thyroxine, phlorhizine, or carbon tetrachloride over periods of a few days gave no change unless used in conjunction with a high fat diet.

Cook (76) has found that 2 per cent of cholesterol in the diet inhibits rat growth, the effect being greater with 15 per cent fat in the diet than with 20 to 30 per cent. The inhibition is due, apparently, to a decreased food intake during the first three weeks. Some 30 per cent of the cholesterol fed was unaccounted for.

The fatty acids of the liver 6 hours after administration of various types of fats have been studied by Peretti (77, 78), who found an increase over the fasting value of from 9.3 per cent with sesame oil to 108.1 per cent with olive oil. Holmgren (79) has observed daily rhythmic variations in liver fat. McHenry (80, 81) finds that vitamin B administered orally causes a marked increase in the liver fat of young rats on a low choline diet; as little as 0.005 mg. daily of crystalline material is effective. Any appetite stimulating effect was eliminated by controlled feeding. The action of the vitamin was apparent at all levels of fat intake. The effect was masked by choline. Koyama (82) observes that starvation causes a greater decrease in the neutral fat fraction of the liver than in the phospholipid. When fatty livers were produced in geese by overfeeding, Flock *et al.* (83) observed that the liver fat was more saturated than normally, it being in fact more saturated than the depot fat. In the spontaneous fatty liver that occurred, together with a high blood fat during the egg-laying season, the iodine number of the liver fat was lower than in the depot fat, although the difference was not as pronounced as after forced feeding. Hilditch & Shorland (84) gave the composition of liver fat in ox, cow, pig, and sheep. They note that the non-phospholipids of the carcass resemble those of the liver except that the latter has more hexadecanoic acid and more of the C_{20} - C_{22} highly unsaturated acids. MacLachlan (85) found no change in fat composition when fatty degeneration was produced by chloroform. Ciaranfi (86) discusses the destruction of butyric acid by liver slices. Dalton (87) finds that free and esterified cholesterol occur in the chick-embryo liver on the eleventh day, and in chorio-allantoic grafts to older hosts on the seventh day. He believes that the esterification of cholesterol occurs in the hepatic cell. Degeneration of the chorio-allantoic grafts occurring on the twelfth day seems to be due to biliary secretion beginning at this time.

METABOLISM OF NITROGENOUS COMPOUNDS

The usual number of suggestions that various phases of amino acid metabolism be used as liver-function tests have appeared in the current literature, but such tests do not seem to have come into general

use. In an extensive report on amino acid and ammonia metabolism in liver disease, Kirk (88) emphasizes that deamination and urea formation are among the last functions to be impaired. In various types of hepatic disease he finds no abnormalities of blood amino acid concentration, amino acid excretion, or urea formation. Blood ammonia may increase above the normal after the oral administration of ammonia compounds in some cases of cirrhosis, but evidence is presented to indicate that this increase is due to entrance of ammonia nitrogen into the general circulation through collaterals that have developed secondary to venous obstruction and is not due to any change in hepatic function. Daft, Robschey-Robbins & Whipple (89) also conclude that urea formation is depressed only by severe or fatal experimental liver injury (chloroform). The concentration of uric acid may be increased by functional liver impairment, as is creatine excretion. Nitzescu & Gontzea (90) have found that dogs with hepatic injury produced by phosphorus exhibit creatinuria and excrete an abnormal amount of creatine when this substance is given by mouth; the same is true of 80 per cent of patients with hepatic disease. Nonnenbruch & Weiser (91) report that the serum residual nitrogen tends to be increased in hepatic disease, and may be lowered by urea administration.

Addis, Poo & Lew (92) find that the livers of albino rats lose 20 per cent of their protein content after a two-day fast, while all other organs combined lose only 4 per cent. They conclude that the liver contains a store of protein which can be drawn upon in time of need. Histological studies by Li (93) confirm the thesis of hepatic protein storage. This author finds that plant (soy-bean) protein gives as much storage as does animal protein. Protein storage in the liver is also supported by Jucker (94), who determined the ratio of purine to total nitrogen and found that it is increased by a protein free diet but not by fasting. Jucker's histological findings likewise indicate a protein reserve that can be given up without injury to the hepatic cell. Luck & Martin (95) find that the amount of albumin extractable from the liver varies with the pH of the solution used for extraction, being less at lower pH values and increasing to a maximum when the pH is 6.3. Scoz *et al.* (96) state that the injection of thyroxin causes first a decrease and then an increase in the protein sulphur of the liver.

Neber (97, 98) discusses the oxidative deamination of amino acids by various tissues, and finds liver the most active. The same

enzyme appears to function in all organs. Heterocyclic amino acids go through a process differing from that of other amino acids. Philpot (99) finds that the oxidase attacking tyramine is of the aerobic type, inhibited by methylene blue. Felix & Derr-Kaltenbach (100) state that the end products of tyrosine breakdown are acetic acid and carbon dioxide; ammonia is not split off. London (101) has continued his experimental injections of amino acid mixtures into the portal vein in an attempt to test the theory of Krebs. Arginine injected into the portal vein causes an output of urea; citrulline and ornithine do not do so. Ornithine injected with ammonium chloride does not increase the amount of urea formed. These findings are considered to be opposed to the theory of Krebs. Hirai (102) finds that the removal of guanidine from the blood stream of rabbits is delayed by hepatic poisons, and the blood guanidine level is increased. It may be questioned whether the method employed does not also determine some creatinine.

GENERAL

Of the many investigations upon hepatic enzymes and enzyme systems, only a few which throw some light upon hepatic metabolism can be mentioned. Hodgson (103) prepared glycogenase from the liver, found its optimum pH to be 8.0, and states that it quantitatively hydrolyses maltose to glucose. The amount in the liver was not decreased by convulsive doses of insulin. Suzuki (104) has studied asparaginase. Mazza (105) investigating the dehydrogenation of phenylaliphatic acids finds a dehydrogenase which transforms hydroxy acids into β -keto acids. He believes this supports the theory of β -oxidation. Bodansky (106) has produced a rise of serum phosphatase by various types of liver injury, and finds that the increased phosphatase may be accompanied by either a rise or fall of serum bilirubin or cholesterol.

Houssay, Marenzi & Gerschman (107) have found that the injection of small doses of epinephrine causes an increase in blood potassium. The potassium originates in the liver, and the same rise in the blood level can be produced by splanchnic stimulation, which, however, is ineffective after ergotamine or hepatectomy. Stimulation of the hepatic nerves will still cause the blood potassium to rise after adrenalectomy. This confirms D'Silva (108), who found that epinephrine liberated potassium from cat liver perfused with saline, the response being greater when blood was used as the perfusate. Kaunitz & Selzer (109) fed high protein diets rich in sodium chloride and

low protein diets low in sodium chloride to rats, and found the liver glycogen, sodium, chloride, and potassium higher in the first instance: there was no change in calcium or magnesium. Tsushima (110) gave 5 per cent sodium chloride solution to rabbits, in some of which liver damage had been produced (phosphorus, India ink, etc.). They found that the sodium chloride content of all tissues and especially that of the liver increased in both normal and poisoned animals, but the livers of the latter retained the sodium chloride for longer periods and reached higher maxima. Shigemi (111), however, found in perfusion experiments that the amounts of various cations fixed by livers damaged with carbon tetrachloride were subnormal. Limited data suggested that hepatic injury increased the water content of liver, kidneys, intestines, and brain. Szanto (112) observed that intradermal saline in rats is more slowly absorbed when much liver tissue has been removed. Lederer & Crandall (113) found the average serum calcium in Eck-fistula dogs to be below normal. They noted that parathormone is less effective in these animals and oral or parenteral calcium seems to be more rapidly removed from the blood. The suggestion is made that this type of liver injury may result in calcium depletion.

Willstaedt & Lindqvist (114) have described two new carotenoids found in liver and serum, which represent, perhaps, oxidation products of β -carotene. Studying the factors producing variations in the vitamin-A content of guinea pig livers, Chevallier (115) observed marked seasonal differences, and states that splanchnic stimulation causes liberation of vitamin A into the blood stream. Chevallier & Choron (116) report that all guinea pigs will store vitamin A in the liver when it is given as such, but when carotene is administered some will store much and others little vitamin. A relationship between hepatic vitamin-A storage and chronaxy has been reported (117). Svirbely (118) finds that cincophen as well as thyroid and dinitrophenol decreases the vitamin-C content of the liver. Rats with fatty livers resulting from carbon tetrachloride still synthesize appreciable amounts of ascorbic acid. The ascorbic acid content of the liver has been shown to be decreased by thyroid administration and increased by thyroid extirpation (119), also decreased by thyrotropic hormone (120) and decreased by the sex hormones (121). Injections of ascorbic acid into non-deficient guinea pigs gave rise to storage in the liver (122). The oxygen consumption of liver slices from scorbutic guinea pigs was found by Stotz (123) to be increased

40 per cent. Heymann (124) produced liver injury in three weeks' old rats on rachitogenic diets by ligation of the common bile duct or by carbon tetrachloride. In such animals 10 to 12 times as much vitamin D given intramuscularly was needed to cure the rickets as in animals on the same diet without liver injury. Heymann concludes that impaired liver function results in decreased effectiveness of vitamin D. Previously, the ineffectiveness of vitamin D in jaundiced animals had been attributed to failure of absorption.⁴ On morphologic evidence Szittay (125) concludes that vigantol in doses sufficient to cause calcification of the vessels increases the metabolism of the liver. Nicolaysen (126) discusses the relationship of vitamin D to the inorganic phosphorus of the liver.

Daft, Robschey-Robbins & Whipple (89), in their studies on liver injury by chloroform, found that intravenous iron gives rise to the expected amount of new hemoglobin whether the liver is normal or damaged. Only with severe or fatal injury is the hemoglobin-forming contribution of the liver, like urea formation, depressed. Hahn & Whipple (127) report that when iron depletion has been corrected, any excess iron administered is stored in the liver. After depletion the iron content of the hepatic parenchyma amounts to 1 to 2 mg. per cent. Wakeham & Halenz (128) found the iron content of blood-free liver in rats to average 10.3 mg. per cent; in anemic rats it amounted to 4.1 mg. per cent. Borgen & Elvehjem (129) have substantiated earlier findings that something less than 70 per cent of the iron in the liver is inorganic. Lesne *et al.* (130) found 14 to 20 mg. of copper per kg. in livers of children less than 2 years of age, and a somewhat smaller amount in older age groups. The iron content was between 0.3 and 0.5 mg. per kg. at all ages. According to Hahn & Fairman (131), the copper content of human liver is within the limits of normal in various pathological conditions. The liver of the dog contains somewhat more copper than does human liver, and in experimental anemia this value increases further. Both Wintrobe (132) and Rosenberg (133) have commented on the occurrence of a pernicious anemia-like picture in patients with hepatic disease. This condition may at times respond to liver extract, and it is suggested that pathological changes interfere with the ability of the liver to store the stroma-building factor.

Knutti *et al.* (134) report that Eck-fistula dogs are remarkably deficient in their ability to form new plasma protein, thus confirming

⁴ Cf. *Ann. Rev. Biochem.*, 5, 446 (1936).

older observations. Gottlieb & Ludwig (135) present evidence for changes in the serum proteins in hepatic disease. Snyder (136) perfused the liver with solutions containing eserine, and found that vagal stimulation caused the liberation of a substance having a vagus-like action upon the heart of a second animal (turtle). This suggests that some liver functions may be under vagal control. Bacq (137) states that the liver and gastro-intestinal tract destroy no more epinephrine than do other tissues. The amount of *p*-bromophenylmercapturic acid synthesized from bromobenzene was found by Stekol (138) to be independent of the time of feeding, suggesting that this function is not subject to the diurnal variations reported for other hepatic activities. By determining the glutathione content of the inflowing and outflowing blood, Binet & Weller (139) have shown that this substance is retained by the liver during fasting and eliminated during digestion. Murata (140) has determined the glutathione content of the liver and other organs, and observed seasonal variations. Fiessinger *et al.* (141) report that when dog liver is perfused with low concentration of alcohol, the alcohol is oxidized. No oxidation occurs, however, in the absence of oxygen or when potassium cyanide is added. Nishimura (142) states that no glucuronic acid is excreted by the dog when camphor is given following a one stage-hepatectomy. Human liver disease is reported to produce abnormalities in the metabolism of oxalic (143, 144) and citric (145) acids.

A number of observations on hepatic blood flow may be mentioned briefly. McMichael (146) finds that the liver obtains two-thirds of its oxygen from the portal vein when the blood pressure is normal; if the blood pressure falls the liver may depend almost entirely on the hepatic artery (cat). In the rabbit most of the oxygen is normally supplied by the hepatic artery. Blalock & Mason (147) have determined oxygen consumption and blood flow in unanesthetized dogs. Franklin & Janker (148) find that the chief outflow from the hepatic vein usually occurs during inspiration. Cherry & Crandall (149) have determined the circulation time through the liver in unanesthetized dogs. Malméjac (150) has made observations on the reservoir function of the liver as determined by splanchnic stimulation. Hasama (151) has recorded an hepatic action current after epinephrine injection or splanchnic stimulation.

BILE

A most interesting monograph on the physiological chemistry of the bile has been prepared by Sobotka (152) and a companion volume

dealing with the chemical and physical properties of the bile acids is in press. The literature is well reviewed up to 1936. A method for the determination of bile salts in bile has been offered by Goiffon *et al.* (153), for desoxycholic and cholic acids by Abe (154), and for the differential quantitative analysis of bile salts in bile by Doubilet (155). Schenk (156) has discussed the chemistry of the oxidation products of the bile acids. Cortese & Bashour (157) have synthesized sodium taurocholate and taurodesoxycholate. Doubilet & Colp (158) have applied the method of the former to the analysis of human fistula bile, and, on release of an uncomplicated obstruction (no infection), find that cholic acid constitutes about 50 per cent of the total bile acids. If inflammation and infection are present cholic acid constitutes about one-sixth of the total, which suggests that this bile acid may be absorbed by the inflamed ducts. In man, Doubilet (159) has found cholic acid more effective than desoxycholic acid in increasing the concentration of bile acids and their total output. In the dog he finds (160) the effectiveness of various bile-salt preparations to fall in the following order: canine bile acids, ox bile salts, glycocholic acid, cholic acid, desoxycholic acid, dehydrocholic acid. Kohlstaedt & Helmer (161) note the constancy of human fistula bile even with variations in diet. Administration of bile salts by mouth causes an increase in their concentration in the bile without a proportionate increase in cholesterol. Surgical procedures depress the bile-salt concentration, and it was observed that the bile salt and cholesterol excretion do not parallel the volume output. Beck (162) fed ox bile to bile-fistula dogs and obtained an increase in volume, decrease in solids, and an increase in pH. There was a good correlation between pH and percentage of solids. Schmidt (163) has studied the effect of thyroxin on the elimination of intravenously injected sodium cholate, and finds that it delays removal from the blood and excretion into the bile, possibly because of liver injury. Virtue & Doster-Virtue (164) found that when cholic acid was given for several days to fasting bile-fistula dogs, the simultaneous administration of cystine (confirmatory of previous investigations) or methionine caused an increase in the taurocholate; homocystine had no effect. The limiting factor in bile-salt production appears to be cholic acid, as has been believed.

Caujolle has found that cobalt (165) and molybdenum (166) are partly eliminated in the bile. Minnhaar (167) has made the same observation for sodium formaldehydesulfoxylate and Loeper *et al.* (168) for ascorbic acid. Bile flow has been found to be greatly de-

creased by an infusion of epinephrine (169) and by parathyroidectomy (170). It is increased by hexamethylenetetramine and decholin (171) and by the addition of raw liver to the diet (172). A generalized alkalosis results in greater alkalinity of the bile (173). Tanturi & Ivy (174) have found that secretin acts as a cholagogue in the absence of the pancreas and intestines.

Of considerable practical as well as theoretical interest are various observations indicating that the liver plays a predominant role in bile-salt formation and destruction, and that relatively slight hepatic damage may produce a profound decrease in the secretion of the bile salts. Bollman & Mann (175), from studies on hepatectomized animals, conclude that the liver is the sole site of bile-salt formation and plays an important part in their destruction. They also show that even such liver damage as is produced by a short period of chloroform inhalation greatly depresses the bile-salt output. Riegel, Ravdin & Rose (176) have observed in the human that both cholesterol and bile-salt concentrations are much decreased by liver disease.

Rothmund (177) has found pyrroporphyrin and traces of coproporphyrin in beef bile, but no evidence for the occurrence of other chlorophyll porphyrins was obtained. Wieland & Hanke (178) state that beef bile contains, in addition to the cholanic acid series, very weak acids of a different type. One of these was purified from 10 tons of bile and found to have the formula $C_{29}H_{46}O_3$; it was termed sapocholic acid. Kishi (179) reports two new bile acids isolated from rabbit bile. According to Baltaceanu & Vasiliu (180), dog gall-bladder bile contains rather more, and liver bile rather less, sugar than does blood; considerable glycoprotein is also present. Oliva *et al.* (181) report the presence of 6.1 mg. of ammonia per 100 cc. of bile, and find that the amino nitrogen of bile is about twice that of blood. Miseta (182) finds 19 to 44 mg. urea and 480 to 560 mg. chlorides per 100 cc. of bile. Free choline, leucine, valine, lysine, and tyrosine have been identified in bile by Müller (183). Mayer (184) has found 8 to 17 mg. per 100 cc. of calcium in liver bile and 44.8 to 53.1 in gall-bladder bile. Jones & Sherberg (185) attempted to find neutral fat or lecithin in gall-bladder bile, and conclude that no more than 0.5 per cent of saponifiable material can be present.

Bashour & Bauman (186) report that the solvent effect of bile salts for cholesterol increases with increasing concentration. Conjugation with amino acids decreases the solvent effect of cholic and deoxycholic acids. Chabrol *et al.* (187) state that the solvent power

of bile for cholesterol is not proportional to the bile-salt content. They therefore feel that some other component of bile must be concerned. Riegel, Ravdin & Rose (188) find that normal human or dog bile hydrolyzes cholesterol esters when mixed with serum; abnormal bile containing esters was not active. Greaves & Schmidt, having found that deoxycholic acid will transport irradiated ergosterol and β -carotene across the intestinal wall, studied (189) taurocholic acid, glycocholic acid, and decholin, and state that these substances are not effective. According to the same authors (190) deoxycholic acid increases the availability of vitamin E for the rat, and they suggest (191) that bile is also necessary for the normal absorption of vitamin K. Shapiro & Koster (192) find that human fecal sterol excretion is decreased when bile is excluded from the intestine. It is suggested that the discrepancy between this observation and those on animals may be explained by the higher cholesterol content of human bile. Heymann (193) has discussed in some detail the differences between biliary fistula rickets and rickets due to other causes. The regressive lesions following total bile stasis in the cat were found by Cantarow & Stewart (194) to be less marked when the animals received dehydrocholate. They suggest the possibility that the changes following obstruction are not due as much to pressure or extravasation of bile as to decreased functional activity of the liver cells. A few of the contributions which deal with the question of cholelithiasis may be cited (195, 196, 197). The evidence seems to favor the view that the pH of bile is of less importance in the etiology of gall stones than the concentration of bile salts or of bile salts and fatty acids.

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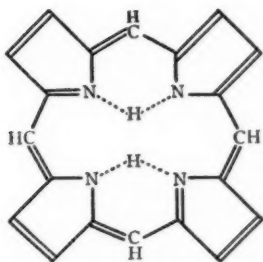
ANIMAL PIGMENTS*

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PORPHYRINS

Porphin.—The basic unit of the porphyrins, porphin (I), has been synthesized by Fischer & Gleim (1) by autocondensation of pyrrole- α -aldehyde and by Rothmund (2) by condensation of pyrrole with formaldehyde.



I. Porphin



II. Phthalocyanine

Azaporphins (Phthalocyanines, Imidoporphyrins, Porphyrines).—Provided that the double bonds of the Küster-Fischer formula of porphyrins (I) have the usual stereochemical properties of double bonds and that the valencies of the pyrrole ring lie in its plane, no other than a completely plane configuration can be ascribed to porphin. Although the formula was generally accepted, the peculiar shape of the molecule had never been confirmed by direct evidence and has not always received due consideration by the biochemist. Conclusive evidence for the plane structure has now been obtained by X-ray studies of phthalocyanine (II), a synthetic substance closely related

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to porphin (3). In the nomenclature recently suggested by Helberger (4), which seems to be the most comprehensive, phthalocyanine is tetrabenzotetraazaporphin. An absolutely direct X-ray analysis of phthalocyanine and its nickel-complex salt has been carried out by Robertson and colleagues (5), phthalocyanine being the first organic substance which has yielded to such treatment. The plane formula of Linstead is fully confirmed. There is no real difference between the four isoindole nuclei, but this is explained by resonance of the double bonds, and is in harmony with chemical evidence in the porphyrin class (nonexistence of isomerides and aromatic character). There is evidence for the existence of two hydrogen bonds between two pairs of the isoindole nitrogen atoms. None of the new conceptions (e.g., electronic or di-radical formulae) is able to give a better account of the fine structure of phthalocyanine as revealed by the analysis of Robertson than the classical formula. The ethylene groups which in the porphyrins replace the benzene nuclei of phthalocyanine will exert a greater disturbing influence on the inner sixteen-membered ring, but the similarity of properties leaves little doubt that the nuclear structures of porphyrins and of phthalocyanine are not essentially different. Porphyrins or porphin apparently have not yet been subjected to X-ray analysis, although well-formed crystals are available.

In the nickel-complex salt the metal atom fills the space in the centre of the molecule and is evenly bound to all four nitrogen atoms, the four linkages between metal and nitrogen and the three linkages of each nitrogen atom being forced into the same plane. The introduction of metal causes, therefore, no appreciable alteration of the molecular volume. The same fact has been observed by Haurowitz (6) for complex salts of porphyrins. Magnetochemical investigations of iron and nickel complex salts of porphyrins and phthalocyanine confirm these results.

Recently several other azaporphins have been synthesized which bridge the gap between phthalocyanine and porphin. β -Substituted mono-, di- and tetraazaporphins (imidoporphyrins) have been obtained by Fischer *et al.* (7). The absorption spectra of these compounds are somewhat similar to those of chlorophyll derivatives (they have in common a $-\text{C}=\text{N}-$ linkage in the sixteen-membered ring not belonging to a pyrrole nucleus). Helberger (4, 8) has synthesized tetrabenzomono-, di- and triazaporphins, while Cook & Linstead (9) have synthesized various tetraazaporphins with and without condensed rings for which they suggest the name porphyrazines.

Absorption and fluorescence spectra.—Stern *et al.* (10) and Dhéré *et al.* (11) have studied exhaustively the spectra of porphyrins and related compounds while those of porphin and methene-substituted porphins have been examined by Albers & Knorr (12). Three types of absorption spectra (aetio-, rhodo- and phyllo-) are distinguished and various rules are found relating the structure of a porphyrin to its spectrum. Hausser, Kuhn & Seitz (13) and Hellström (14) have made the first attempts to bring the frequencies of the fluorescence and absorption bands of porphyrins into a level system. Although their measurements and systems do not quite agree, in both the Raman frequency of the $\text{—C}=\text{C—}$ linkage ($\nu = 1560 \text{ cm}^{-1}$) plays an important role. The fluorescence of a porphyrin is minimal at its isoelectric point and the pH-fluorescence curve (180) is characteristic and may even be used to distinguish between isomeric porphyrins.

Uroporphyrins.—Porphyrins belonging to this group are neither tetramethylporphintetramethylmalonic acids, nor tetramethylporphintetrasuccinic acids as previously assumed. Porphyrins of these structures have been synthesized (15) and have been shown to differ from uroporphyrins (16). The eight carboxyl groups of uroporphyrins are probably contained in four acetic acid and four propionic acid side chains (17). The dualism of porphyrins of type I and type III (order of side chains in type I, $a b a b a b a b$; in type III $a b a b a b b a$), established at first for the coproporphyrins, has recently been proved for the uroporphyrins as well. Uroporphyrin III has been isolated from the urine of patients with acute porphyria by Waldenström (18), by Mertens (19), and by Hoesch & Carrié (20). Chromatographic analysis has shown that uroporphyrin III accompanies the predominant uroporphyrin I in the urine of patients with chronic porphyria (16) and of cattle with ochronosis, a hereditary disease very similar to human chronic porphyria (21).

Biological origin.—It is impossible to discuss here more than a small fraction of the great amount of work done in the field of physiology and pathology of the porphyrins during the last few years. The dualism of the porphyrins is of great importance in the problem since a transformation of blood pigment (a derivative of type-III porphyrin) into porphyrins of type I is unlikely. From the writer's investigations on the mechanism of bile-pigment formation it has become evident that porphyrins are not normal intermediates in haemoglobin disintegration. They may be intermediates in blood-pigment formation. Porphyrins have been found in the albumen of

the hen's egg at a stage of development in which it cannot be derived from the much smaller amount of haemoglobin present in the embryo at that time (22). The observations (23) that porphyrin is present in the embryo and in erythroblasts (Borst & Königsdörfer, 181), that the porphyrin content of reticulocytes is larger than that of erythrocytes, and that porphyrin I excretion is increased with increased haematopoietic activity of the bone marrow (24) support this view.

Pathological amounts of porphyrins may arise in three different ways: (a) By faulty porphyrin synthesis, porphyrins of type I are built which do not combine with iron *in vivo*. Fink has established the important fact that the porphyrin present in small amounts in normal urine is mainly coproporphyrin I (25). In the fox squirrel (*Sciurus niger*) a physiological porphyria exists, uroporphyrin I being deposited in the bones and excreted in the urine (26). The mechanism determining the synthesis of type-III porphyrins is not quite selective under normal physiological conditions and may be further deranged by inborn errors of metabolism, by sulphonal, or by other still unknown factors. Every increase of haematopoietic activity will also cause a proportional increase in porphyrin-I synthesis. There is no evidence that the formation of porphyrins occupies an earlier place in evolution than that of haematin compounds (theory of atavistic porphyrin formation of H. Fischer). (b) A disturbance in the combination of porphyrin of type III with iron may lead to the excretion of free porphyrin III. Rimington assumes this to be the mechanism of formation of coproporphyrin III in lead intoxication. (c) A chemical aberration from the normal haemoglobin-breakdown processes may cause the formation of porphyrins of type III instead of bile pigments. Evidence for the existence of such an aberration, already mooted by Garrod, has been brought forward by Schreus & Carrié, and it is used to explain the increased porphyrin excretion in lead intoxication and in fever, infections, and liver diseases (27, 28). The porphyrin excreted in the majority of the latter cases is, however, coproporphyrin I (29), and the mechanism assumed by Schreus can explain only the formation of porphyrins of type III (in some cases of acute porphyria, of liver cirrhosis, and liver tumour, and perhaps in lead, salvarsan, and some alcohol intoxications). Carrié in his book (28) reviews the methods of clinical porphyrin investigations.

Porphyrins in bituminous products.—The remarkable stability of porphyrins is revealed by their discovery in petroleum, asphalt, coal, and bituminous shale, the most ancient of these sediments

being Silurian (30). Although most of these porphyrins by their constitution reveal derivation from chlorophyll, some are of animal origin (mesoporphyrin, mesoetioporphyrin, deuterioetioporphyrin). The porphyrins in these sediments occur partly free and partly bound to metal as haemochromogens and vanadium complexes. The formation of the vanadium complexes must be a secondary process since no vanadium-porphyrin compound is known to occur in living animals. Important geological conclusions as to the source of the sediments and the conditions of their formation can be drawn from the nature of the porphyrins present.

HAEMATIN COMPOUNDS

Linkage of haematin to iron and nitrogenous substances.—The problems of the nature of the iron-porphyrin linkage in haematin compounds and of the linkage between haematin and nitrogenous substances are so interwoven that they must be discussed together. In the instance of some metal compounds (notably those of iron, cobalt and nickel) it can be decided by measurements of their magnetic susceptibilities whether the metal is bound by ionic or by covalent linkages. Whereas the ions of these metals possess free unpaired electrons in sub-shells, causing paramagnetic susceptibilities, the entry of electrons into these shells, connected with the formation of covalent linkages, makes some or all of the unpaired electrons disappear and causes diamagnetic properties or a decrease of the paramagnetic susceptibility of the complex. The nickel salt of mesoporphyrin ester and nickel phthalocyanine are diamagnetic (31) and nickel is bound in these complexes to four nitrogen atoms by covalent linkages. The more complicated situation with regard to the iron-porphyrin compounds has been elucidated by Pauling & Coryell (32). In haem (heme, reduced haematin, ferroheme¹), the iron-porphyrin complex corresponding to nickel-porphyrin in composition, the iron is not bound by covalent linkages, but is held in essentially ionic combination, since four unpaired electrons are indicated by the magnetic behaviour of the substance. The haemochromogens (ferrous hemochromogens), however, are diamagnetic and the iron in them is linked by covalent bonds to the four porphyrin nitrogens as well as to the two nitrogens of the nitrogenous compound with which

¹ This and the following parenthetical terms have been suggested by Pauling & Coryell.

haem is combined in haemochromogens. This explains what hitherto was difficult to understand, namely the fact that the absorption spectrum of haem is quite different from those of the corresponding complex salts of other metals, while haemochromogens in spite of their different composition have a very similar absorption spectrum. In haematin (ferriheme hydroxide) and haemin (ferriheme chloride) the iron is held in ionic combination. Since haemin in pyridine solution gave the same magnetochemical results as haemin in alkali, pyridine-parahaematin has iron in an ionic state, in contrast to haemochromogen, and, therefore, the suggestion of Pauling to replace the name parahaematin by "ferric hemochromogen" does not appear suitable. The nitrogenous substance in parahaematin cannot be bound to the iron by covalent linkage; it is probably attached to it by dipole linkage or it is attached to the porphyrin nucleus as a molecular compound.

Still more complicated is the situation with regard to the haemoglobin derivatives (32, 33). Oxyhaemoglobin and carbon monoxide-haemoglobin are diamagnetic; in reduced haemoglobin (ferrohaemoglobin), however, the iron is in an essentially ionic state. When oxygen combines with haemoglobin, not only the unpaired electrons of the iron atom, but also those of the oxygen molecule disappear, and the electronic structure of oxygen as well as of haemoglobin undergoes a considerable alteration. The remarkable ease with which bond types can be changed has been observed so far only in haematin compounds and is probably of great importance for their biological functions. The four haem groups in haemoglobin appear to have magnetic moments almost entirely independent of one another. Incidentally these results offer a decisive proof that globin is bound to the iron atom in oxyhaemoglobin, and this is probably true also for the paramagnetic haemoglobin compounds, although the type of linkage is different. Cyanmethaemoglobin (ferrihaemoglobin cyanide) and sulfhydryl-methaemoglobin (ferrihaemoglobin hydrosulphide) contain covalently linked iron (one unpaired electron per haem); acid methaemoglobin (ferrihaemoglobin) and fluormethaemoglobin (ferrihaemoglobin fluoride) contain iron in an ionic state (five unpaired electrons per haem), while in alkaline methaemoglobin (ferrihaemoglobin hydroxide) there are three unpaired electrons per haem, and four covalent bonds resonating between the six adjacent atoms (four porphyrin nitrogens, globin nitrogen, and oxygen of the hydroxyl). The equilibrium between acid and alkaline methaemoglobin is written:

$(\text{HbOH}_2)^+ \rightleftharpoons (\text{HbOH}) + \text{H}^+$ [cf. also Haurowitz (34)], and the results of the magnetochemical titration agree with those of the spectrophotometric study of the same equilibrium by Austin & Drabkin (35). The ferrous compounds with covalent linkage of iron (haemochromogens, oxyhaemoglobin, carbon monoxide-haemoglobin) have an absorption spectrum with two distinct bands, while those with ionic linkage of iron (haem, haemoglobin) have only one band. The spectra of carbon monoxide-haemoglobin and carbon monoxide-haemochromogens are not significantly different (36). In the ferric series the relation between absorption spectrum and structure is less clear. The spectra of the ferric cyanide compounds are quite different from those of the compounds with other residues and cyanmethaemoglobin, cyanpara-haematins and, in the visible part of the spectrum, cyanhaematin have practically the same absorption spectra (37). According to Hogness, Zscheile, Sidwell & Barron (38) cyanhaematin is $[\text{haem}(\text{CN})_2]^-$ [cf. also Haurowitz (71)], but if a similar structure is to be given to cyanmethaemoglobin, the globin cannot be combined to the iron.

From investigations on the equilibrium between haematin and cyanhaematin the same authors conclude that haematin is dimeric. The hydroxyl group of haematin appears to be similar to that in ferric hydroxide. Two crystalline anhydrides have been obtained by Hamsik (39). Chlorine, bromine, thiocyanogen, acetyl, and formyl are also bound heteropolarly in the corresponding haemins (40).

For the consideration of the haem-protein linkage the peculiar flat shape of the haem molecule has to be kept in mind. The large plate of this molecule can be bound to a protein either by one valency, covering the surface of the protein molecule, or if bound by two valencies (as in the haemochromogens), haem must be inserted in crevices of the protein molecule. To the first class of compounds belong haemoglobin, catalase and peroxidase, none of which yields haemochromogen on reduction unless the protein part of the molecule is denatured. This type of linkage leaves one place of coordination free for the attachment of oxygen (in haemoglobin) or of hydrogen peroxide (in catalase and peroxidase) and is probably essential for the biologically important properties of these compounds. Residual forces of globin causing its association in solution are screened off by its combination with haem (41). The greater degree of regular orientation connected with denaturation of a protein probably produces the crevices in which haem can be bound by two nitrogen atoms

in juxtaposition to form a haemochromogen, and the same arrangement must be present in the protein-like substances to which haem is bound in cytochrome-*c* or in heliocorubin. The resistance of cytochrome-*c* (42) and of heliocorubin (43) to oxidation is perhaps due to steric hindrance caused by the embedding of haem in the protein molecule, although denatured globin-haemochromogen is autoxidizable.

Haemoglobin.—Only a few papers on haemoglobin can be considered. Bergmann & Niemann (44) have determined the percentage of those amino acids in the hydrolysate of ox haemoglobin and other proteins for which exact analytical methods are available and have come to conclusions of considerable significance for the chemistry of proteins. The number of molecules of the individual amino acids in proteins can be represented by the formula $2^m \cdot 3^n$, where m and n are whole numbers. The total number of amino acids in haemoglobin is $2^8 \cdot 3^2 = 576$. Ox haemoglobin contains three molecules of cysteine (plus cystine), twelve each of arginine, proline, and tyrosine, sixteen of glutamine, thirty-two each of histidine and aspartic acid, and thirty-six of lysine. The authors represent the protein molecule by a number of superimposed frequencies referring to position numbers of each amino acid. When these are arranged according to their respective frequencies the structural pattern will only begin to repeat itself after 576 units are present, and this offers a chemical explanation for the rule of Svedberg (45) that the molecular weight of proteins is often a simple multiple of 17,500 ($2^4 \cdot 3^2$ amino acid units).

The haemoglobins of various animals differ in the number of cysteine molecules (two, three, six) that they contain. Horse blood appears to contain two haemoglobins with different sulphur content (46), and while the haemoglobin of human individuals belonging to blood groups A and B has fourteen sulphur atoms, that of individuals of blood group O contains only thirteen (47). In contrast to earlier workers Mirsky & Anson (48) found reactive sulfhydryl groups in haemoglobin. At pH 6.8 such groups are present only in denatured globin, but in more alkaline solution, more and more are formed in native haemoglobin up to a maximum at pH 9.5. The sulfhydryl groups and ferrous ion of haemoglobin can be oxidized separately, the former by cystine at pH 9.5, the latter by ferricyanide at pH 6.8. Haemoglobin is the only protein which takes up more iodine, as iodine bound to carbon, than corresponds to the tyrosine, and Bauer & Strauss (49) ascribe this to a varying integral part of the histidine

residues reacting as tyrosine. By action of strong solutions of urea, acetamide, and formamide, horse haemoglobin dissociates into particles of molecular weight 34,500 (50), but in spite of its identical molecular weight the new haemoglobin is not myoglobin. The Soret band of methaemoglobin (at 410 m μ) is completely restored when a solution of "acid haematin" obtained by splitting methaemoglobin at pH 3.0 is brought back to pH 4 to 5 (51). Holden considers this process as a renaturation of a reversibly denatured globin, similar to that previously reported by Anson & Mirsky after salicylate denaturation. The Soret band is shown by all porphyrin compounds but is shifted towards the ultraviolet and decreased in intensity by association of the haematin molecules in solution (52). This band is not found in the absorption spectrum of an erythrocyte suspension because haemoglobin combines loosely with stroma protein to a compound which does not possess this band (53).

The kinetics of the oxidation of haemoglobin to methaemoglobin by molecular oxygen indicates that oxygen reacts with reduced haemoglobin (54). The methaemoglobin formation in metabolizing tissue, however, is due to the action of hydrogen peroxide formed by aërobic dehydrogenase systems (55), although not all phenomena of this reaction can yet be explained satisfactorily. The reactions of haemoglobin with nitric oxide and sodium nitrite have been studied (56, 57).

Ferguson (58) has adapted the chemical method of Ferguson & Roughton for the determination of carbhaemoglobin (haemoglobocarbamic acid and its salts) for the study of the reaction of human haemoglobin with carbon dioxide. There is a nearly linear relation between the carbhaemoglobin content and the degree of reduction of haemoglobin; as much as 30 per cent of the carbon dioxide transported by the blood and 75 per cent of that transported by the erythrocytes is in the form of carbhaemoglobin.

The existence of specific differences between foetal and adult haemoglobins (Barcroft) has been confirmed for goats, cattle and rabbits by the study of the resistance towards alkali and of the spreading velocity (59). An alkali-resistant haemoglobin appears again in human blood at the age of three years accompanying the alkali-labile haemoglobin of early childhood. The dissociation of oxyhaemoglobin in erythrocytes and in dilute solution has been compared by Hill & Wolvekamp (60). Its kinetics have been studied by Pauling (61). Anions have a considerable influence on the dissociation of oxyhaemoglobin (62).

Muscle haemoglobin (myoglobin).—In contrast to the S-shaped dissociation curve of oxyhaemoglobin, that of myoglobin is hyperbolic and is little influenced by pH (63). Millikan (64) has measured the reaction velocities of myoglobin with oxygen and carbon monoxide by means of the Hartridge-Roughton streaming fluid apparatus. Myoglobin reacts much more rapidly with both gases than haemoglobin, and carbon monoxide-myoglobin also dissociates more rapidly than carbon monoxide-haemoglobin; the dissociation velocities of oxymyoglobin and oxyhaemoglobin are approximately the same. The rapid reaction with oxygen makes myoglobin very suitable as an intracellular indicator of oxygen tension with a time lag of less than one-hundredth of a second. By photoelectric spectroscopy of mammalian muscle *in situ* with intact blood and nerve supply, Millikan has proved that myoglobin acts as a short-time oxygen store in the muscle to tide it over periods of oxygen want in each contraction. Carbon monoxide-myoglobin is much more soluble in strong ammonium sulphate solution at pH 6.6 than carbon monoxide-haemoglobin, and this property can be used for separation of the two substances (65). The myoglobins of different animals are species-specific like their haemoglobins.

Erythrocruorins.—Since the haemoglobins of invertebrates differ from those of vertebrates in amino acid composition and in isoelectric point, independently of their molecular weight, they have been separated from the haemoglobins under the name erythrocruorins (Svedberg). Their protein bears a close resemblance to that of chlorocruorins, having a large content of arginine (about 10 per cent) and less (about 5 per cent) histidine and lysine (66). The erythrocruorins dissolved in the haemolymph have a high molecular weight (>360,000), while those enclosed in red cells have a low molecular weight (17,000 to 68,000). The individual differences in the erythrocruorin series are even greater than those in the haemoglobin series.

Catalase.—Crystalline catalase has been obtained from ox liver by dioxane extraction and ammonium sulphate precipitation (67). The view of Zeile that catalase is a ferric protohaematin-protein compound is now established. Crystalline catalase contains 0.1 per cent iron, indicating that the minimum molecular weight is 55,000. The catalase factor is given as 43,000 as in Euler's best preparation.²

² The higher values given by Keilin & Hartree were caused by a mistake in calculation and by the use of \log_e instead of the customary \log_{10} .

Keilin & Hartree (68) have found a strict proportionality between the absorption band at 630 m μ of horse-liver catalase and its activity. The catalase of erythrocytes is spectroscopically identical with that of horse liver (68, 92). Various compounds of catalase with other substances are described by Keilin & Hartree and by Stern (69), of which catalase-ethylhydroperoxide and azide-catalase-hydrogen peroxide, red compounds with a two-banded absorption spectrum, are the most interesting. There can be little doubt that they are closely related to the substrate-enzyme compound, catalase-hydrogen peroxide, the breakdown of which is too rapid to be observed. Keilin assumes his catalase-hydrogen peroxide compounds to be ferrous, since their absorption band is shifted by carbon monoxide, and hydrogen peroxide causes a reduction which hyposulphite is unable to produce. The similar hydrogen peroxide compounds of methaemoglobin (70, 71), of peroxidase (55), and of haematin (72, 73) are, however, ferric compounds. Evidence for the formation of ferrous haem-hydrogen peroxide compounds as intermediate products in the oxidation of protohaematin to verdohaematin has been brought forward by the writer (93). Whereas some inhibitors of catalase (hydrogen cyanide and hydrogen sulphide) prevent the formation of the hydrogen peroxide compound by combining with catalase, others (sodium azide, hydroxylamine and hydrazine) inhibit the action of the enzyme by stabilizing the hydrogen peroxide compound. The identity of the prosthetic group of catalase with protohaematin IX has been established by Stern (74). Haemoglobin, catalase, and probably peroxidase, differ only in their specific proteins, which exert a fundamental influence on the biologically important properties of these compounds. The reversible splitting of catalase into haematin and the protein carrier by dialysis against dilute hydrochloric acid, claimed by Agner, has not been confirmed (75).

Peroxidase.—The nature of peroxidase from horseradish and milk as a protein-haematin compound has been proved by Keilin & Mann (76). The absorption spectrum in slightly acid solution resembles that of acid methaemoglobin with a band at 645 m μ the strength of which is proportional to the enzyme activity, whereas the total haematin content of the preparations is larger and not proportional to enzyme activity. Towards alkali and towards reducing agents, peroxidase behaves similarly to methaemoglobin. Its haematin appears to be protohaematin. The formation of two different compounds of peroxidase with hydrogen peroxide (enzyme-substrate

compounds) and their subsequent disappearance in the reaction with various substrates of peroxidase can be observed with the spectro-scope. The enzyme combines reversibly with sodium fluoride, potassium cyanide, hydrogen sulfide and nitric oxide, and with azide in the presence of hydrogen peroxide, to form spectroscopically different compounds, the existence of which explains the inhibiting action of these substances on the activity of the enzyme. The peroxidase of fig sap has been purified by Sumner & Howell (77) and has been shown to be a combined haematin.

Cytochrome-c.³—The preparation of cytochrome-*c* has been simplified by Keilin & Hartree (42). The nature of the prosthetic group of cytochrome-*c* is not yet established. Although Zeile had shown in 1932 that the "haematin-*c*" arising by repeated alternating reduction and oxidation of haemochromogen is different from cytochrome-*c* haematin in spite of its spectroscopically similar behaviour (Keilin), several authors have recently again assumed that the two substances are identical (78, 79). The experiments of Zeile have been confirmed by the writer (92), and Roche & Bénévent (80) have shown that the product of oxidation of protohaemochromogen is unable to give a haemoglobin with native globin, while cytochrome-*c* haematin and the haematin of Zeile⁴ give haemoglobins. It is, therefore, probable that the haematin of cytochrome-*c* is derived from addition of a molecule of water to one and of a tertiary base and water to the second vinyl side chain of protohaematin, but a definite proof is still lacking. Such a structure would account for the very basic isoelectric point of cytochrome-*c* (81).

By digesting haemolyzed erythrocytes of various animals with pepsin-hydrochloric acid a water-soluble, ether-insoluble, and ultra-filtrable haematin-*c* was obtained by Barkan & Schales (82). This cannot be the haematin of cytochrome-*c* since it is not transformed into haematoporphyrin by hydrogen bromide-glacial acetic acid. The authors assume that it is present in the blood as a haemoglobin-*c*, but its formation from protohaematin by condensation with protein split products does not appear excluded.

Cytochrome-a and related compounds.—Cytochrome-*a* is characterized by an absorption band at 604 mμ and cytochrome-*a*₁ by a band at 586 mμ. Both give a pyridine-haemochromogen with an ab-

³ Cf. *Ann. Rev. Biochem.*, **5**, 9 (1935); **6**, 31 (1937).

⁴ Cf. *Ann. Rev. Biochem.*, **3**, 441 (1934).

sorption band at about 584 m μ . From heart muscle Negelein (83) had isolated a haematin which gave a crystalline haemochromogen with a single absorption band in the visible at 587 m μ . According to Roche & Bénévent (84) this haematin is not the true haematin of cytochrome-*a*. These authors obtained by a similar method a haematin-*a* which differed from Negelein's compound by giving a haemochromogen with two absorption bands (582 and 530 m μ). On standing in aqueous acetone the second band disappears and the compound of Negelein is formed. This arises also by slow autoxidation of haemin dissolved in pyridine. Both haematin-*a* show the absorption band in the violet, characteristic of porphyrin compounds (at 425 m μ).

There is good evidence in favour of the formation of compounds of this type by oxidation of protohaematin. Warburg's respiratory ferment (indophenol- or cytochrome-oxidase) and the *Spirographis* haematin belong to the same class of compounds. The constitution of *Spirographis* haematin has been elucidated by Fischer & Seemann (85) as that of the iron complex salt of 1,3,5,8-tetramethyl-2-formyl-4-vinylporphin-6,7-dipropionic acid. Like all other haematin derivatives found in nature, it belongs to the porphyrin-III type and differs from protohaematin only by a formyl group replacing the vinyl group 2, whereas in the somewhat similar haematin from chlorophyll-*b*-porphyrins, formyl replaces a methyl group. The oxidation-reduction potentials of *Spirographis* haemochromogens are somewhat higher than those of the corresponding protohaemochromogens (86). *Spirographis* haematin or the next higher homolog with acetyl instead of formyl may be derived from protohaematin by an oxidation which leaves the porphyrin nucleus intact but oxidizes a vinyl side chain, whereas in the oxidation of protohaematin to verdohaematin leading ultimately to bile pigment the side chains are left unaltered and the porphyrin nucleus is oxidized. Roche & Bénévent considered the haematin-*a* as the intermediate step of verdohaematin formation. This is hardly likely and the experiments of Lemberg, Cortis-Jones & Norrie (93) indicate that the conditions under which haematin-*a* and those under which bile-pigment haematin is obtained from protohaematin, exclude one another. While haematin-*a* is formed by the action of oxygen or hydrogen peroxide on protohaematin in the presence of metal-free cysteine and of glutathione (slowly without and rapidly with copper), i.e., under conditions in which haematin remains in the ferric state, verdohaematin (or its precursor) is formed when the haematin iron is kept reduced by ascorbic acid

or by cysteine-copper. Lwoff (87) has shown that some micro-organisms (flagellates and bacteria of the *Haemophilus* class) synthesize respiratory ferment and cytochrome from protohaematin, which they require as a growth substance; added cytochrome-*c* cannot fully replace protohaematin. The amount of respiratory ferment and cytochrome-*a* is reduced in milk-anaemic rats and is restored by the addition of copper but not of iron, and addition of copper to the medium of yeast cultures causes increased production of cytochrome-*a* (88). The glutathione-copper mechanism of protohaematin oxidation may, therefore, occur *in vivo*.

In certain bacteria, notably *B. coli*, cytochrome-*a*₂ with an absorption band at 628 mμ is observed. This is probably a bile-pigment haemochromogen (92). The compound with an absorption band at 639 mμ, observed by Warburg, Negelein & Haas (89) when *B. pasteurianum* is shaken with air in the presence of cyanide is, according to Lemberg, Cortis-Jones & Norrie (93), an oxyporphyrin-haematin compound, formed only under the conditions of Warburg's experiment by oxidation of protohaematin. It is a precursor of bile-pigment formation.

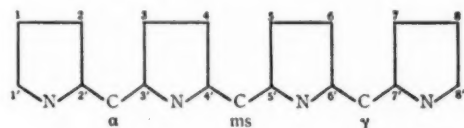
BILE PIGMENTS

The formation of bile pigments from blood pigments.—Recent investigations have lent strong support to the hypothesis of the writer (90, 91) that the intermediate stages of the physiological bile-pigment formation are not haematin and porphyrin, but bile-pigment haemochromogens and biliverdin.⁵ Verdohaemochromogens occur in nature, e.g., in preparations of horse-liver catalase, of cytochrome-*c*, and in erythrocytes (though in small concentration) (92). The mechanism of the formation of verdohaemochromogens by the coupled oxidation of haemochromogens and ascorbic acid has been studied in detail by Lemberg, Cortis-Jones & Norrie (93). A ferrous haem-hydrogen peroxide compound is formed first, the oxygen is then transferred to the α -methene group, forming the haematin of a chlorin-like compound, probably an oxyporphyrin, and this methene group is then removed and the ring split open by the action of oxygen, verdohaematin being formed. Haemoglobin reacts with ascorbic acid and oxygen in a similar way, though more slowly (94), and the "green pigment" thus obtained is transformed by acid into biliverdin (95)

⁵ Cf. *Ann. Rev. Biochem.*, **5**, 479 (1936).

[cf. also (96)]. Several different bile-pigment haemochromogens, varying with the conditions of preparation, are contained in the "green pigment" (92). Iron is easily detached from verdohaemochromogen and it was, therefore, suggested by the writer that the "easily detachable blood iron" of Barkan is of bile-pigment-haematin nature (92). Independently Barkan & Schales (97) have arrived at the same conclusion. By the action of hydrogen peroxide in the presence of cyanide they have obtained from haemoglobin " α -pseudohaemochromogen," a compound similar to but different from verdohaemochromogen, possessing the characteristic properties of "easily detachable blood iron." The same compound can be observed spectroscopically among the products of the action of ascorbic acid and oxygen on haemoglobin and in a small concentration in normal erythrocytes (98). The formation of α -pseudohaemochromogen occurs apparently in the erythrocytes themselves. Whether this new haematin compound is a bile-pigment haemochromogen and the role it plays in bile-pigment formation remain to be investigated.

Various bile pigments of the meso-type have been synthesized by Siedel (99, 107) by condensation of α -hydroxy- α' -formyl- or of α -hydroxy- α' -hydroxymethyl-dipyrromethenes or the corresponding dipyrromethanes with α -unsubstituted α' -hydroxy-dipyrromethenes or dipyrromethanes, e.g. mesobiliverdin (glucobilin) and mesobilirubin. The syntheses confirm the constitutions ascribed to them. A new systematic nomenclature of bile pigments is suggested: The nuclear system of mesobilinogen is termed "bilan"; the rubins are "bilidiens," the verdins "bilitriens," and the position of the double bonds and side chains is indicated according to the scheme:



The view of the writer that the unsaturated side chains in bilirubin and biliverdin are vinyl groups as in haemin (90) appears to be no longer contested by Fischer [cf. (85), p. 157].

By ultracentrifuge and cataphoretic methods Pedersen & Waldenström (100) have confirmed the earlier findings of Bennhold that both "direct" and "indirect" bilirubin of the blood serum are bound to serum albumin. Bilirubin combines readily with serum

albumin but not with ovalbumin. The well-known diazo reaction of bilirubin is not given by bilirubin itself, but by a dipyrrole decomposition product (101). Naumann (102) has demonstrated the occurrence of small amounts of bilirubin in normal urine. A new method for determination of bilirubin in blood serum, which avoids the losses caused by protein precipitation, has been described by Malloy & Evelyn (103).

Urobilins and urobilinogens.—The isolation of crystalline mesobilinogen (mesobilirubinogen) from some samples of pathological urine by Fischer in 1911 has led to the mistaken generalization that urobilinogen is identical with mesobilinogen. It is evident today that mesobilinogen prevails only in pathological urines (104); in the writer's experience its predominance even in pathological urines is rare. The urobilinogen of urine is in general identical with stercobilinogen though it may contain small amounts of mesobilinogen. The identity of the urinary urobilin with the stercobilin of faeces, isolated in 1932 by Watson, has been established by Watson (105) and by Heilmeyer & Krebs (106). Stercobilin is related to stercobilinogen, and "urobilin IX α " to mesobilinogen, as dipyrromethenes are to dipyrromethanes. The use of the names "natural urobilin" for urobilin IX α or of the name urobilinogen for mesobilinogen is, therefore, misleading. In the writer's opinion the term urobilin ought to be given to stercobilin, and another name ought to be sought for urobilin IX α . This substance has been synthesized by Siedel & Meier (107) and the synthesis has proved the formula with the CH-group in the middle of the molecule, suggested by the writer in 1934 (108). Stercobilin (C₃₃H₄₆O₆N₄) differs from urobilin IX α (C₃₃H₄₂O₆N₄) by having two pyrroline nuclei (I and IV) instead of pyrrole nuclei. By the action of concentrated sulphuric acid stercobilin is transformed to mesobiliverdin (109). This proves definitely that it does not contain more oxygen than urobilin IX α , as was previously assumed (105, 108). In contrast to all other bile pigments stercobilin is optically active (110) with a remarkably strong optical rotation which is easily destroyed by hydrogenation or the formation of the zinc-complex. It can also be distinguished from urobilin IX α by its resistance to oxidation and by the situation of the absorption band in acid solution (108, 111). The presence of small amounts of urobilin in normal human blood serum has been established by Heilmeyer & Ohlig (112).

"Pentdyopent" is the name given by Bingold (113) to a substance

first observed by Stokvis, which after heating with alkali and reduction with hyposulphite displays an absorption band at 525 μ . This substance accompanies bilirubin in urines containing bile pigment and in gall stones; it occurs in small quantities in the erythrocytes and in urine of healthy subjects (113, 114). Bingold has shown that it is formed by the action of hydrogen peroxide on haematin or bile pigments. Pentdyopent is closely related to α, α' -dihydroxydipyrromethenes (115). It constitutes a product of further oxidation of bile pigment.

Bile-pigment chromoproteins.—Pigments of this type, similar in constitution to those discovered by the writer in phycoerythrin and phycocyan of *Rhodophyceae*, have been found to occur in butterfly wings, accompanying pterins (168)). Biliverdin (oocyan) occurs in eggshells of the cassowary, to a great extent as chromoprotein (116).

HAEMOCYANINS^o

It is still undecided whether the copper in haemocyanins is bound to an actual prosthetic group of peptide nature (haemocuprin) or whether this is a mixture of accidental scission products of the polypeptide chain containing the copper atom. The fact that the haemocuprins of Schmitz, of Conant, and of Roche & Dubouloz (117) are different supports the second view. The haemocyanins of different animals differ in their solubility in salt solutions (118). Svedberg (45) has given a comprehensive survey of his researches on the molecular weights of proteins by ultracentrifuge methods, including a review on the molecular weights of haemocyanins and their dependency on pH. Ultrafiltration experiments give results similar to those obtained with the ultracentrifuge (119). The pH-stability range of *Helix* haemocyanin in aqueous solution (pH 4.5 to 7.3) does not include the physiological pH, but calcium and magnesium ions extend it over this zone towards the alkaline side (120). Cyanide reacts with oxyhaemocyanin so as to remove the oxygen, forming a stable cyanhaemocyanin which contains two $-\text{CN}$ groups per copper atom (121).

CAROTENOIDS

The reader is referred to a number of reviews on the subject (122 to 126). Only those carotenoids are discussed here in greater

^o Cf. *Ann. Rev. Biochem.*, 5, 470 (1936).

detail which are specific products of the animal body and not taken over from plants. While the storage fat of some animals (pig, dog, rodents) does not contain any carotenoids, some species take up selectively carotene hydrocarbons (horse, cow), other species xanthophylls (birds) and man and frog both kinds of substances, though the body fat of the frog contains a very high content of carotene only (127, 128, 129). The carotenoids of human serum have been studied by several investigators (130 to 133). Since human serum and fat contain lycopene and capsanthin in addition to carotene, colorimetric determinations without previous chromatographic separation yield carotene values which are too high.

*Astacin.*⁷—Karrer, Loewe & Hübner (134) have shown astacin to be 3,4,3',4'-tetraketo- β -carotene.⁸ Its composition, $C_{40}H_{48}O_4$ (not $C_{27}H_{32}O_3$ as found by R. Kuhn), follows from the analysis of its dioxime. It contains two pair of α -diketo groups, forming a diquinoxaline derivative, and two of these keto groups are enolizable, which explains the acid properties of astacin and the existence of a non-acidic diacetyl derivative. Further facts which support the formula are the results of hydrogenation, of Zerewitinoff determinations, and of the oxidation of the diquinoxaline derivative which leads to α,α -dimethyl-succinic acid. The epiphasic ester (astacein) is the dipalmitic ester; the hypophasic ovo-ester is an ester with a fatty acid of six to eight carbon atoms without hydroxyl or amino groups (135).

Visual purple.—There is now convincing evidence that visual purple (rhodopsin) is a carotenoid-albumin. Brunner, Baroni & Kleinau (136) obtained β -carotene from the retinas of pigs and steers by the action of methyl alcohol under nitrogen. β -Carotene adsorbed to alumina shows a red colour and an absorption band at 500 m μ similar to that of rhodopsin. By the action of chloroform Wald (137) obtained, however, a yellow ligroin-soluble substance with no definite absorption bands, retinene, shown by an antimony-chloride compound with an absorption band at 666 m μ to be related to the carotenoids. This substance plays an important part in the visual cycle. Visual purple first is bleached by light to an unstable orange compound, "transient orange," which in a dark-process changes to "indicator yellow" (138, 139). "Indicator yellow" appears to be

⁷ Cf. *Ann. Rev. Biochem.*, **3**, 420 (1934).

⁸ In the older nomenclature 4,5,4',5'-tetraketo- β -carotene.

a loose protein compound of retinene; it is obtained from rhodopsin also by the action of acid or alkali. Retinene breaks down further to give vitamin A, a process which in the dark is accompanied by partial resynthesis of visual purple. It was known that vitamin A occurs in the retina (Wald) and that its lack causes night-blindness and disappearance of visual purple from the retina. In the eye, not in the isolated retina, visual purple is resynthesized from vitamin A. The pigmented layers of the eye contain, in addition to xanthophyll esters (probably biologically inactive), a considerable store of vitamin A which migrates from the retina into these layers in the bleaching process, and back into the retina for rhodopsin synthesis in the dark adaptation. The bleaching process does not require oxygen (140).

Visual purple has the properties of an albumin (141). Mirsky assumes the transformation of visual purple to visual yellow to be a reversible protein denaturation (142) by light, the colour component sensitising the protein to visible light. Exceptional in this process is the low activation energy, for which one quantum of visible light suffices.

Whereas rhodopsin is found in mammals, birds, amphibians and some marine fishes, fresh water fishes contain a different visual purple with an absorption band at 525 m μ for which the name "porphyropsin" is suggested (143). It enters a visual cycle similar to that of rhodopsin, but the compounds obtained differ from retinene and vitamin A. The colourless compound is characterized by an antimony-chloride compound with an absorption band at 693 m μ (vitamin A₂). In some marine fishes other related compounds (or mixtures of porphyropsin and rhodopsin) occur (143, 144). From the cones of chicken, pigeon, and turtle eyes Wald & Zussmann (145) have isolated astacin, xanthophyll esters, and a yellow-green hydrocarbon, contained in the cones in separate red, orange-yellow, and yellow-green oil globules which are probably important for colour discrimination.

FLAVINS⁹

Until now only one flavin, lactoflavin (6,7-dimethyl-9-*d*-1'-ribityl-isoalloxazine) has been found to occur in nature, either free (e.g. in cow's milk), in the form of its phosphoric acid compound cytoflav (in liver and heart), or as "yellow enzyme." In human milk lacto-

⁹ Cf. *Ann. Rev. Biochem.*, 5, 8, 361 (1936); 6, 342 (1937).

flavin appears to be present in loose combination with protein (146). Lactoflavin and the products of its decomposition by light (lumiflavin and lumichrome) have been isolated from ox retinas (147) where they appear to play a role in the synthesis of visual purple from vitamin A (148). Kuhn, Rudy & Weygand (149) have synthesized lactoflavin-5'-phosphoric acid through the 5'-trityl compound. The synthetic compound is split by α -glycerophosphatase as rapidly as cytoflav (150) and combines with the protein carrier to form active "yellow enzyme." Karrer, Frei & Meerwein (151) are not convinced that this synthesis proves the structure of the natural flavin phosphate, but its behaviour in the oxidation by periodic acid also shows that the position of the phosphoric acid group is 5'. Cytoflav may be present in the tissues as nucleotide combined with adenylic acid. No less than three compounds exist between flavin and leucoflavin (which has two more hydrogen atoms), one of which is a radical having paramagnetic properties, and the other two molecular compounds of this radical with flavin and leucoflavin (152).

Although only one flavin occurs in nature, several others can be synthesized which are also biologically active. The flavins with a glycosido group at 9 cannot be reduced by the animal body and are biologically inactive (153). Only *d*-ribityl-flavin and *l*-arabityl-flavin are active as coferments (active groups) of the yellow enzyme and as vitamin B₂ (154, 155). The activity of *d*-arabityl-flavin reported by Euler, Karrer & Malmberg (156) was caused, according to Kuhn & Weygand (157), by a molecular rearrangement in the sugar molecule in the condensation with the aromatic amine, leading to the *d*-ribityl compound.

The effect of the methyl substituents has been studied by Kuhn and by Karrer (126, 155, 158, 159, 160). Alkyl in 6 or 7 (methyl, ethyl or 6,7-cyclotrimethylene or cyclotetramethylene rings) appears to be necessary for biological activity which is suppressed by methylation at 3,5, or 8. An unsubstituted imino group at position three, and perhaps also definite steric arrangements of the sugar hydroxyls, are required for the combination with the protein carrier, while the methyl groups on the benzene ring exert a great influence on the redox potential (161) and together with the hydroxyls of the sugar side chain diminish the toxicity (155, 162).

The process for the purification of "yellow enzyme" has been simplified and extended (163, 164). By dialysis against 0.02 *N* HCl the yellow ferment is split and the protein carrier is denatured (free

sulfhydryl groups), but the protein is renatured by subsequent dialysis against water (165). The carrier possesses the properties of an albumin and its amino acids consist of 25 per cent of bases with predominance of lysine (164). Lactoflavin combines loosely with the carrier by its imino group at position 3, but lactoflavin phosphoric acid is bound much more firmly. From cataphoretic experiments on "yellow ferment" and on the carrier, Theorell concludes that the carrier is bound by a basic protein group to the phosphoric acid group and simultaneously by an acidic protein group to the basic imino group at 3. In the writer's opinion, however, this imino group has an acidic, not a basic, character. At 37° and pH 5 the carrier loses its power of recombining with flavin phosphate. This process, which is also reversible, is not a proper denaturation (free sulfhydryl groups do not appear). The inactivation is probably due to a distortion of the protein causing the two groups of the protein necessary for the combination with flavin phosphate to be shifted from the suitable steric position. This appears to be a striking example of the lock-key mechanism suggested by E. Fischer, with the difference that the specific linkage is here between active group and carrier, not between enzyme and substrate.

PTERINS

Schöpf & Becker (cf. 166) have isolated from insects (167) several new pterins which are closely related to xanthopterin ($C_{19}H_{18}O_6N_{16}$) and leucopterin ($C_{19}H_{19}O_{11}N_{15}$). Wieland & Kotzschmar (168) have obtained several new products of partial breakdown of leucopterin, but no definite conclusions as to the structure of the compound are yet possible. Three double bonds in the molecule, similar to the 4,5-double bonds of uric acid, are indicated by the reaction with chlorine which introduces three diglycol groups: three guanine-like parts of the molecule are suggested by the hydrolysis leading to three molecules of guanidine.

While Hopkins saw in the pigments of the butterfly wings an example of the use of an excretory substance for ornament, recent investigations show rather that they are one more example of the use of a physiologically important substance for this purpose. Koschara (169) has discovered uropterin (probably identical with xanthopterin) in normal human urine and has shown that pterins are widely distributed in nature, though in very small concentration. Pterins are not excretory products, for birds and snakes, as well as those insects

which display pterins in their wings or on their body, excrete only uric acid and no pterins (170). Recent investigations (171) suggest that pterins play a role in erythrocyte formation.

MISCELLANEOUS

Vitamin P, which in co-action with ascorbic acid regulates vascular permeability, has been recognized as a mixture (called "citrin") of two flavanone glucosides, hesperidin and eriodictyol glucoside (172, 173).

By the action of the cytochrome-indophenol oxidase system and of a hydrogen-cyanide-insensitive system present in heart and skeletal muscle, adrenaline is oxidized to adrenochrome, the *o*-quinone of 1-methyl-3,5,6-trihydroxy-2,3-dihydroindole (174). Adrenochrome acts as hydrogen carrier from lactic and malic dehydrogenase systems to oxygen.

Blount (175) has isolated lanigerin, $C_{17}H_{14}O_5$, a polyhydroxyanthraquinone, from woolly aphis (*Eriosoma lanigerum*) and strobilin ($C_{30}H_{24}O_8$), probably a polyhydroxyphenanthraquinone, from white pine chermes (*Adelges strobi*).

Chromatographic analysis becomes increasingly important in the study of natural pigments. Several reviews on this method are available (176, 177, 178). Apart from its application to the study of carotenoids, where it is the standard method, it has been employed in the study of porphyrins (16, 18, 21, 30), bile pigments (99), and urinary pigments (pterins, flavins and others) (18, 169, 179).

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THE TERPENES, SAPONINS, AND CLOSELY RELATED COMPOUNDS*

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AND

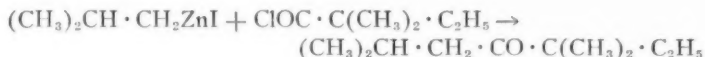
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Four years have passed since the last review of this subject, during which there has been an accumulation of roughly 500 papers that have been reviewed in chemical abstracts. Because of the very limited space allotted, it has been possible to select, perhaps somewhat arbitrarily, only what appears to us to be of general interest and to have reached a stage of elucidation or at least convergent views.

MONOTERPENES

The structure of the open chain artemisia ketone (II), from the ethereal oil of *Artemisia annual*, interesting because it represents the only naturally occurring aliphatic terpene built up from an irregularly assembled isoprene chain, has been definitely established by the synthesis of its tetrahydro derivative (I), by Ruzicka, Reichstein & Pulver:



I



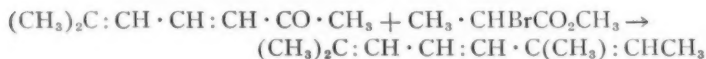
II

This confirms the formula for artemisia ketone proposed by Asahina & Takagi in earlier work but for which there was no proof in regard to the left half of the molecule in Formula II. Ruzicka *et al.* point out that although in the majority of cases there is a regular arrangement of the isoprene units in the terpenes or polyprenes there are still many instances of an irregular arrangement and therefore

* Received January 17, 1938.

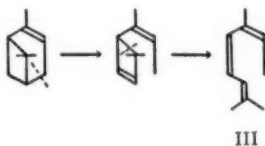
great caution is to be observed in premature *a priori* assumptions of such a regular arrangement in interpreting structure.

The formation of dipentene and a diterpene along with various aromatic hydrocarbons on pyrolysis of α -pinene is a matter of record. Arbuzov has recently noted that when α -pinene is passed over copper-chromium or cobalt-thorium catalysts at 340–400° up to 25 per cent of acyclic alloocimene (III) is formed, in which the temperature rather than the catalyst appears to play the main role. The latter substance, obtained by boiling the unstable natural terpene, ocimene, has been synthesized by Fischer & Löwenberg by use of the Reformatsky reaction followed by decarboxylation and dehydration.



III

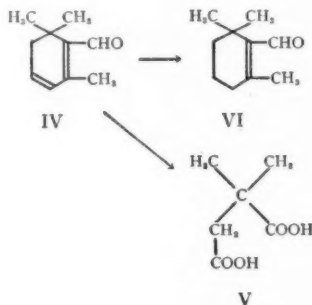
The course of the pyrolysis is represented as follows:



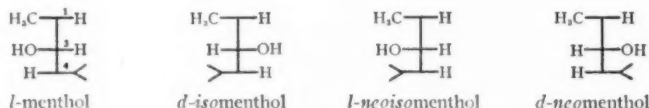
III

The remaining problems connected with the chemistry of the terpene glucoside picrocrocin have been solved by Kuhn & Winterstein. The formula has been readjusted to $\text{C}_{16}\text{H}_{26}\text{O}_7$, and its carbonyl group has been found to be aldehydic. Despite its glucosidic nature, hydrolysis into an ethereal oil, $\text{C}_{10}\text{H}_{14}\text{O}$, called safranal (IV), and glucose can be effected not only by acid, but preferably by alkali, a fact which left two problems unsettled, *viz.*, the structure of safranal and the manner by which it is linked with glucose. The former has been answered by oxidative degradation to *as*-dimethyl succinic acid (V), and by partial catalytic hydrogenation to β -cyclocitral (VI). Picrocrocin then is derived from safranal by addition of glucose to one of its double bonds. The latter can be only in the $\Delta^{4,5}$ position since absorption spectrum observations on picrocrocin show an ethylenic double bond to be conjugated with the carbonyl group. The apparent failure of water to enter into the hydrolysis must be due to elimination of the glucosidic hydroxyl group during hydrolysis. Re-

duction of the $\Delta^{2,3}$ double bond renders alkaline hydrolysis impossible. Picrocrocin is pictured as existing in the form of a hypothetical bicyclic carotenoid pigment "protocrocin," consisting of a crocin chain to either end of which a molecule of picrocrocin is joined. This is supported by the relative molecular amounts of the bitter principle of picrocrocin and the dye crocin (1.4:1) found in fresh saffron.



Read and his coworkers have continued their investigations on the stereochemistry of the menthols and menthones. Reinvestigation of the four isomerides of the former by Read & Grubb has led them to advance configurations for the menthols and menthylamines which represent a modification of earlier schemes. By comparing the relative velocities of reaction of the alcohols with various nitrobenzoyl chlorides under the assumption that steric hindrance, as first suggested by Vavon & Couderc, is the determining factor in such esterifications, the hydroxyl group is now placed *cis* to the isopropyl group in the less reactive *neomenthols*, and the following configurations are thus assigned:



These configurations differ from those assigned by Zeitschel & Schmidt, which were based mainly on the easier dehydration of *neomenthol* to *menthene*. Read & Grubb interpret this as due to a *trans* elimination of water. Their conclusions are supported by the results

of a study of the relative rates of esterification of the monobenzoates of the *cis*- and *trans*-cyclohexane-1,2-diols as noted by Wilson & Read. The *trans* form is more easily esterified. Read & Grubb also interpret steric hindrance as the chief influence in determining other distinguishing reactions in this series, such as the predominance of *neomenthol* in the reduction products of *menthone*.

Read and his coworkers have commenced a similar series of studies in the *carvone* series and although important observations have been made the work has not progressed to the stage reached in the case of the *menthols*.

The catalytic reduction of *diosphenol* has also been reinvestigated by Walker & Read and observations have been made which show that this substance may be used as a "turntable" between the *menthone* and *carvomenthone* series. Since *diosphenol* is a natural product this may have a biological significance.

Claim to the first complete synthesis of *dl*-*piperitone* has been recorded by Walker by the condensation of β -chloroethyl methyl ketone with ethyl sodio- α -isopropylacetoacetate to ethyl Δ^1 -*p*-*menthen*-3-one-4-carboxylate and subsequent hydrolysis.

The question of the nature of the decomposition products of *camphor* in the animal organism and of the substance responsible for the cardiotoxic effect of so-called "vita camphor" has occasioned considerable work. Asahina & Ishidate (1) showed that the "campherol" of Schmiedeberg & Meyer consists principally of 3-hydroxy- and 5-hydroxycamphor together with smaller amounts of the hitherto unknown *cis*- and *trans*- π -hydroxycamphors, these substances being eliminated in the urine as glucuronides. Tamura and coworkers in a series of pharmacological studies have indicated that *d-trans*- π -oxocamphor is responsible for the cardiotoxic effects observed. *Trans*- π -oxocamphor has been prepared by Asahina & Ishidate (2) by the catalytic reduction of *trans*- π -apo-7-camphoryl chloride by the Rosenmund method, and showed cardiotoxic activity in dogs. However, presumably because of the ease with which it undergoes auto-oxidation, it has not been isolated as such from the urine of camphorized dogs. Asahina & Ishidate (2) have strengthened Tamura's hypothesis by the isolation of the oxidation product of *trans*- π -oxocamphor, *trans*- π -apo-7-camphoric acid, from the urine of camphorized dogs. In the course of their study of the isolation of pure *p*-diketocamphane from campherol they encountered also α - and β -santenone. A clue to the origin of the latter two substances was obtained by Asahina &

Ishidate (2) from the interesting observation of Reinartz, Zanke & Faust that 5-oxocamphor is transformed by the animal organism into π -hydroxycyclocamphanone. It appeared possible that, by a similar transformation, camphor is changed to teresantalol, from which the santenones are formed during the production of diketocamphane and subsequent isolation process. Reinartz, Zanke & Schaefer found that *epicamphor* like camphor is also oxidized in the dog to hydroxy camphors. These were found to be principally 4-hydroxy*epicamphor* and in slight amount π -hydroxy*epicamphor*, which are excreted in the urine as glucuronides.

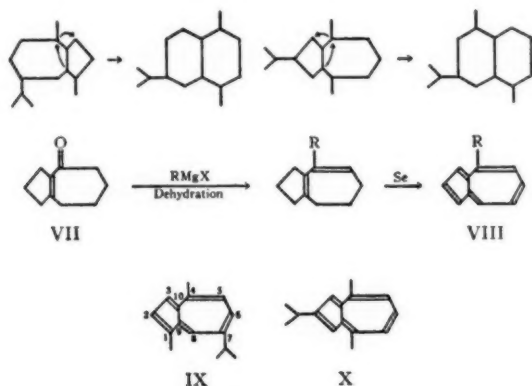
SESQUITERPENES

The chemistry of the hitherto comparatively obscure "azulenes" has been considerably advanced. Pfau & Plattner (1) in a careful reinvestigation showed that contrary to the earlier views the number of blue hydrocarbons, $C_{15}H_{18}$, obtained by sulfur or selenium dehydrogenation of various essential oils may be materially decreased. Eucazulene, from eucalyptus oil, and gurjunazulene, from gurjun balsam, are identical with guajazulene, from guajol, the only azulene resulting from dehydrogenation of a chemical individual of known structure. However, a new and different azulene, vetivazulene, was obtained from vetiver oil.

The same workers have attacked the problem of the constitution of the hydrocarbons. The extremely unsaturated character of the azulenes renders the results of vigorous oxidative degradation difficult of interpretation. Therefore a different procedure was employed. Treatment of guajol and vetiver oil, the precursors of guajazulene and vetivazulene, with hydriodic acid and phosphorus followed by sulfur dehydrogenation confirmed an observation of Komppa on ledol (ledene) (which had given cadalene as well as azulene) that naphthalene derivatives may be produced. From the former 1,4-dimethyl-6-isopropyl-naphthalene and from the latter 1,5-dimethyl-7-isopropyl-naphthalene were isolated. Pfau & Plattner (1) point out that the assumption that the azulenes possess a naphthalene skeleton is untenable on a number of grounds, and that such a skeleton arises by a rearrangement of the original ring system.

Pfau & Plattner (1) announced that oxidative rupture of one ring of the precursor sesquiterpenes and subsequent reclosure led to a cyclic ketone containing one less carbon atom than the original. Catalytic dehydrogenation of the latter substance produced a phenol

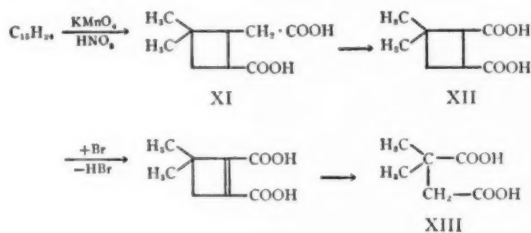
from which the deduction would appear to follow that the azulenes contain a basic seven-membered ring. Similar degradation made probable the presence also of a five-membered ring. The formation of the above naphthalene derivatives is postulated on the basis of a type of retropinacoline rearrangement:



With the idea of the presence of a seven-membered ring in mind, Pfau & Plattner (1) turned to the synthetic approach. Starting with the cyclopentano-cycloheptenone of Hückel & Schnitzspahn (VII), a number of 4-substituted derivatives have been prepared which on selenium dehydrogenation furnished the corresponding hydrocarbons (VIII), containing five double bonds. These manifested striking similarity in properties (solubilities, color) to the azulenes derived from natural sources. In a later paper Plattner & Pfau have prepared the basic unsubstituted hydrocarbon, bicyclo-0,3,5-decapentaene (1,3,5,7,9), for which the generic term "azulene" is proposed. The structures assigned to guajazulene and vetivazulene then are Formulas IX and X.

Progress has been made in the rather involved chemistry of the Caryophyllenes, the mixture of hydrocarbons occurring in oil of cloves, and one of the most widespread of the naturally occurring sesquiterpenes. Earlier investigations, particularly by Deussen, had shown the oil to consist of three principal components, α -, β -, and γ -caryophyllene, of which the α -isomer is presumably identical with humulene from hop oil. Since it is impossible to separate the hydrocarbons themselves from the mixture by physical means, well-defined

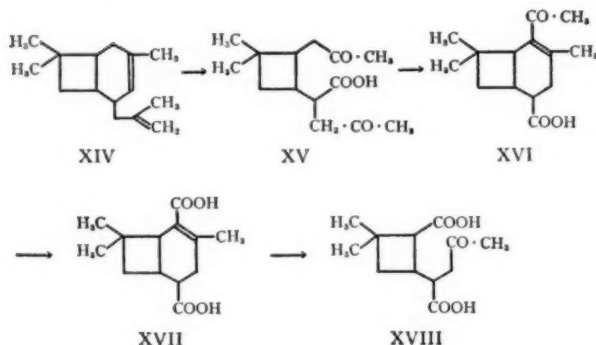
crystalline derivatives have also been employed for preliminary separation and degradation purposes. By vigorous oxidation of γ -caryophyllene, from the mother liquor of the blue nitrosite of β -caryophyllene, Evans, Ramage & Simonsen obtained a crude caryophyllenic acid which was separated through the ester with subsequent hydrolysis into *as*-dimethyl succinic acid, *cis*-caryophyllenic acid (XI), and *d-cis*-norcaryophyllenic acid (XII). Caryophyllenic acid had previously been assumed by Semmler & Mayer to be homogeneous. Similar work had been done in part previously and then simultaneously by Ruzicka & Zimmermann. Ramage & Simonsen (1) succeeded in degrading XI to XII which on ring cleavage yields *as*-dimethyl succinic acid (XIII) (Evans, Ramage & Simonsen; Ruzicka, Zimmermann & Huber):



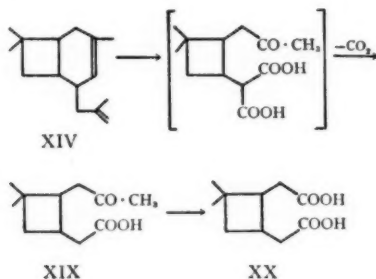
The above structure of *d-cis*-norcaryophyllenic acid, first suggested by Ruzicka & Zimmermann, has been confirmed by synthesis by Rydon. This work has been complicated by the encountering also of the *trans* forms of these acids.

Ruzicka & Wind, and then Ruzicka, Zimmermann & Huber have furnished the evidence for the most probable structure of the chief constituent of the caryophyllene mixture, β -caryophyllene (XIV). This hydrocarbon on partial ozonization gave the diketone acid (XV) of Semmler & Mayer. On treating the ester of this acid with alcoholic alkali, ring closure occurred to give the keto acid (XVI). The methyl ketone group of (XVI) was degraded with hypobromous acid to give the acid (XVII) which suffered ring cleavage on ozonization leading to the dibasic keto acid (XVIII).

Since the latter when heated loses one molecule of water but no carbon dioxide, the positions of the side chains should be as indicated in Formula XIV. Corroborative evidence has been obtained by Ruzicka and coworkers by complete oxidative degradation of caryo-



phyllene which led ultimately to homocaryophyllenic acid (XX), presumably by way of the keto acid (XIX) (the $C_{11}H_{18}O_3$ keto acid of Semmler & Mayer).



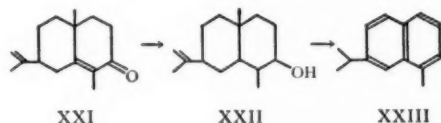
Compound XX has been synthesized by Ramage & Simonsen (2). Although both the diketo acid (XV) and the monoketo acid (XIX) have been simultaneously obtained from a supposedly homogeneous β -caryophyllene nitrosite by Ramage & Simonsen (3), it is still possible, as discussed by Ruzicka *et al.*, that XIX is derived from γ -caryophyllene which may differ from β -caryophyllene only in regard to the position of the double bond of the side chain (an isopropenyl instead of the isopropylidene group of the former).

The process by which caryophyllene is transformed into tricyclic clovene as well as the structure of the latter remains unsettled. Blair has proposed a structure for clovene based on a caryophyllene structure derived from a folded farnesol chain. However, as Ruzicka

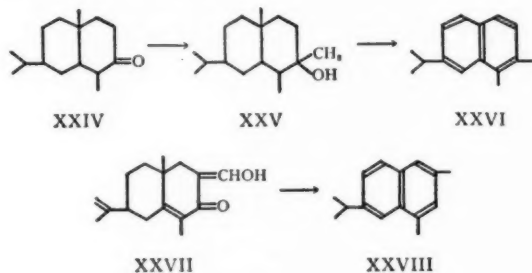
points out, such a structure for caryophyllene does not agree with the degradation results and cannot be accepted.

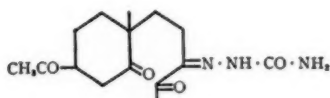
Homocaryophyllenic acid has also been obtained by energetic oxidation of betulol [Treibs; Ramage & Simonsen (2)]. However, the tentative structure for this sesquiterpene alcohol as advanced by Treibs remains to be rigidly confirmed.

Hedge & Rao found that the higher boiling fractions of the oil of *Cyperus rotundus* consist principally of a sesquiterpene ketone, α -cyperone. The study was continued in the later work of Bradfield, Hedge, Rao, Simonsen & Gillam. This ketone was readily isomerized to β -cyperone. The former (XXI) can be easily isolated as the semicarbazone, and on reduction with sodium and alcohol yields dihydro- α -cyperol (XXII), showing one double bond to be conjugated with the carbonyl group. XXII on selenium dehydrogenation gave eudalene (XXIII).



Tetrahydro- α -cyperone (XXIV), on reaction with methylmagnesium iodide, yields a methyl carbinol (XXV) which on dehydrogenation gives 1,2-dimethyl-7-isopropyl naphthalene (XXVI), thereby locating the carbonyl group. Finally, the nuclear double bond is placed on the basis of the formation of a hydroxymethylene derivative of XXI, *viz.*, XXVII. The latter, after reduction followed by dehydrogenation with selenium, yields 1,3-dimethyl-7-isopropyl naphthalene (XXVIII) (Bradfield, Pritchard & Simonsen):

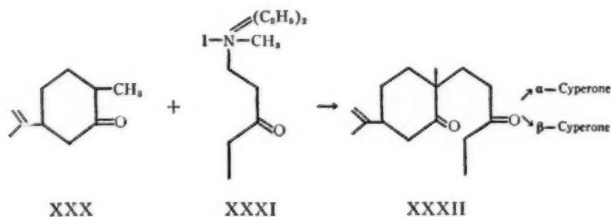




XXIX

Since the semicarbazones of both α - and β -cyperone yield the same ozonization product (XXIX), the two ketones are presumably stereoisomerides.

These structures have been confirmed by synthetic evidence (Bradfield, Jones & Simonsen; Adamson, McQuillan, Robinson & Simonsen) of which space permits but limited discussion. *l*-Dihydrocarvone (XXX) condenses with the methiodide of *l*-diethylaminopentane-3-one (XXXI) in the presence of sodamide to give XXXII. The latter on ring closure with sodium ethoxide yields α -cyperone and with 50 per cent sulfuric acid β -cyperone.



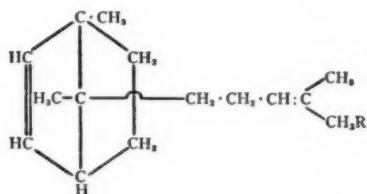
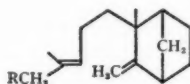
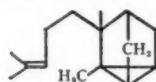
XXX

XXXI

XXXII

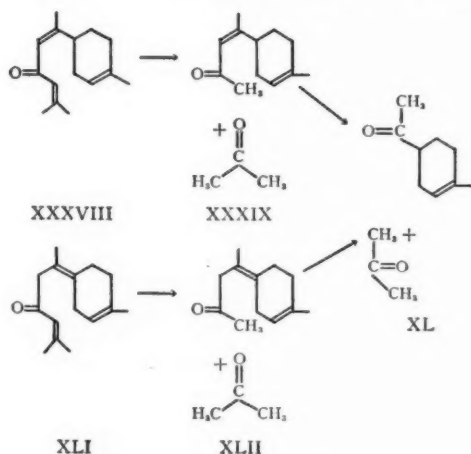
A renewed attack on the problem of the structures of β -santalol and β -santalene has been made. Bradfield, Penfold & Simonsen isolated pure α - and β -santalol from the oil of *Santalum album*. Without going into detail these workers have advanced Formulas XXXIII and XXXIV as best representing β -santalol and β -santalene. Ruzicka & Thomann, however, favor the structures XXXV and XXXVI for these substances, and have discussed their relationship to the substances of the α series (XXXVII).

Two monocyclic sesquiterpene ketones, α - and γ -atlantone, have been isolated from Atlas and Himalaya cedar oil by Pfau & Plattner (2). α -Atlantone, which is given the structure of an α,β unsaturated ketone (XXXVIII), is triply unsaturated and on treatment with alkali splits to acetone and acetyldipentene (XXXIX). Further treatment with alkali cleaves a second acetone residue from XXXIX, yielding acetylmethylcyclohexene (XL). γ -Atlantone (XLI), the iso-


 XXXIII. R = OH
 XXXIV. R = H

 XXXV. R = OH
 XXXVI. R = H


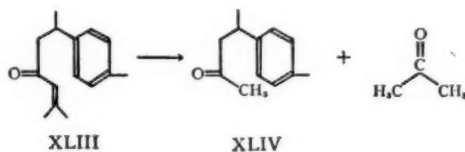
XXXVII

meric Δ^8, γ -ketone, on the other hand, on similar treatment yields XL via acetylterpinolene (XLII). Reduction of the carbonyl group in XXXVIII and XLI, respectively, by the Wolff-Kishner method led to α - and γ -bisabolene, both of which yield the same trihydrochloride.

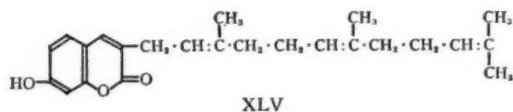


The odoriferous constituent of turmeric oil has been shown by Rupe, Clar, Pfau & Plattner to be a mixture of at least two ketones, *ar*-turmerone (XLIII), a benzenoid sesquiterpene ketone, and turmerone, together with very small quantities of the atlantones. The

structure of the former was clearly demonstrated by its hydrolysis with alkali to curcumone (XLIV), the structure of which had previously been shown by Rupe & Wiederkehr. Turmerone is a triply unsaturated sesquiterpene ketone, the carbon skeleton of which is identical with XLIII. The position of the double bonds, two of which are apparently nuclear, remains to be definitely settled.

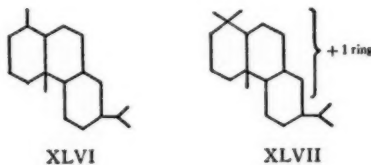


Space does not permit detailed discussion of the interesting straight chain sesquiterpene derivative, ammosesinol, for which the structure XLV has been advanced as a result of the separate investigations of Kunz, Späth, and their coworkers.



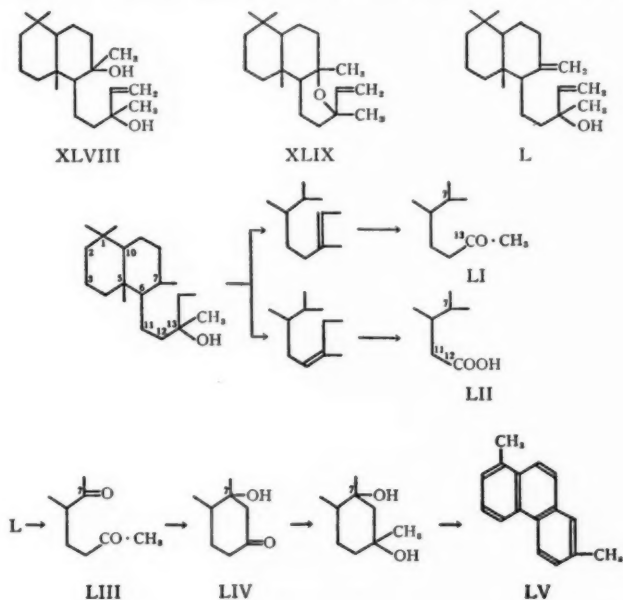
DITERPENES

Several communications deal with various diterpene derivatives. Ruzicka & Waldmann pointed out that the lignin hydrocarbon, fichtelite, hitherto regarded as a perhydroretene, $C_{18}H_{32}$, could be derived biogenetically from abietic acid, which would necessitate revision of the empirical formula to $C_{19}H_{34}$ by virtue of the presence of the angular methyl group. Careful dehydrogenation experiments wherein one mole of methane was found in the gas evolved confirmed this idea and fichtelite now is assigned the structure XLVI by analogy to abietic acid.



The members of an interesting group of previously described lignite hydrocarbons have been shown to be identical with each other and with certain new plant hydrocarbons. Briggs (1) isolated a hydrocarbon, $C_{20}H_{32}$, from the leaf oils of *Phyllocladus alpinus* and *Araucaria excelsa* which proved to be identical with the phyllocladene first described by Baker & Smith, with dacrene (Blackie), and with sciadopitene isolated from *Sciadopitys verticillata* by Nishida & Uoda. Phyllocladene is tetracyclic and probably contains the phenanthrene structure together with an additional 3- or 4-membered ring and one double bond. Dihydrophyllocladene has been shown by Briggs (2) to be identical with the lignite hydrocarbon, iosene, of Soltys. Soltys obtained retene on selenium dehydrogenation of iosene. The structure XLVII has been suggested as conforming to the isoprene rule and as falling in line with abiatic acid and fichtelite. The position and the nature of the fourth ring are unknown.

A study of the dicyclic diterpene resins from New Zealand pines has furnished confirmatory evidence for the constitution assigned to the monoethylenic tertiary diol, sclareol (XLVIII), by



Ruzicka & Janot. A singly unsaturated manoyl oxide, $C_{20}H_{34}O$ (XLIX), and the doubly unsaturated tertiary alcohol, manool, $C_{20}H_{34}O$ (L), were isolated by Hosking & Brandt from *Dacrydium colensoi* and *D. biforme*, respectively. Selenium dehydrogenation of XLIX gave 1,2,5-trimethylnaphthalene (agathalene) and 1,2,8-trimethylphenanthrene, indicating a carbon skeleton similar to that of agathenic diacid and sclareol. Ethereal hydrogen chloride easily opens the oxide ring of XLIX, yielding the same trihydrochloride as that obtained from XLVIII and L. XLIX and L thus are respectively cyclic ether and dehydration products of XLVIII. Dehydration of tetrahydromanool yields a mixture of isomeric hydrocarbons, which on ozonization gives the ketone, LI, and the acid, LII, showing the position of the hydroxyl group of L. Ozonization of manool leads to the 1,5-diketone (LIII). LIII readily undergoes an internal aldol condensation to give LIV, which after conversion to the tertiary alcohol by treatment with methylmagnesium iodide, followed by dehydration and selenium dehydrogenation, yields pimanthrene (LV). From this it follows that a methylene group is attached to C_7 in L. In XLVIII one of the tertiary hydroxyl groups and in XLIX the oxide bridge are attached to this carbon atom. The three substances represent a diterpene group analogous to the group α -terpineol, terpinhydrate, and cineol.

From the resin of *D. colensoi*, 3-ketomanoyl oxide was also isolated and was converted into XLIX by reduction by the Wolff-Kishner process. The position of the carbonyl group was shown by dehydrogenation of the carbinol formed by the action of methylmagnesium iodide to 1,2,5,7-tetramethylnaphthalene and presumably 1,2,6,8-tetramethylphenanthrene.

TRITERPENES

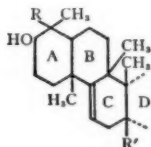
During the time elapsed since the last review considerable activity has continued and definite progress has been made in the extremely involved and difficult field of the triterpenes. Special emphasis has continued to be placed on the importance of the three methods of attacking the problem of structure, *viz.*, degradation, dehydrogenation, and the application of the isoprene rule. The brilliant work of Ruzicka and his coworkers has been in largest part responsible for the recent progress and they have relied most on dehydrogenation studies (interpreted in accordance with other degradation results and the isoprene rule). This work has been done principally with the so-

called acid sapogenins: hederagenin, oleanolic acid, gypsogenin, sumaresinolic acid, etc.; although Ruzicka and coworkers have very recently found a similar investigation of the amyrins and erythrodiol among other neutral triterpenes to be rewarding. Kitasato has also made important contributions by the study of the oxidative degradation of hederagenin and oleanolic acid.

Of special importance and interest is the precise correlation of a number of these triterpenes already indicated to be closely related by previous studies. Ruzicka & Giacomello (1) first showed that gypsogenin, as obtained by hydrolysis of the saponin of white soap root, was not homogeneous and that a purification could be effected as the acetyl derivative. Then they obtained oleanolic acid from gypsogenin acetate by replacing the carbonyl group of the latter by hydrogen with simultaneous saponification of the acetyl group, thus at the same time establishing the formula of gypsogenin definitely as $C_{30}H_{48}O_4$. The carbonyl group of this sapogenin was then (2) shown to be aldehydic in character by oxidation to a carboxyl group and also by catalytic reduction to the primary alcoholic group. In the latter case the reaction product was found to be hederagenin. A similar correlation of the dihydric triterpene alcohol erythrodiol was accomplished by Zimmermann by the oxidation of a primary alcoholic group to carboxyl with the formation of oleanolic acid. And now only within the past month Ruzicka & Schellenberg have announced the simultaneous conversion of oleanolic acid into β -amyrin and erythrodiol. This was accomplished by reduction of oleanolyl chloride by Rosenmund's method to the aldehyde and subsequent reduction of the latter by the Wolff method to a mixture of β -amyrin and erythrodiol. Thus these five triterpenes must agree not only as regards their gross structure but also in stereochemical configuration. They differ only in respect to the make-up of two side chains. These relationships will be clearly seen by reference to the partial formula LVI given below and to the table for the nature of R and R' in each case. However, a difference of opinion persists in regard to the position of the group R', as will be noted in the subsequent discussion.

In general, the more recent views have continued to accept as preferable the hydropicene ring system, especially in the case of those triterpenes which yield on dehydrogenation the 1,8-dimethylpicene melting at 306° , the identity of which has just been determined by Ruzicka & Hofmann. It has been suggested, although without supporting evidence, that such a picene ring system could possibly

R	R'	
CH ₃	CH ₂ OH	Erythrodiol
CH ₃	COOH	Oleanolic acid
CH ₂ OH	COOH	Hederagenin
CHO	COOH	Gypsogenin
CH ₃	CH ₃	β-amyrin

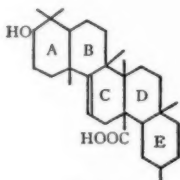


LVI

result from partial rearrangements during dehydrogenation. In addition, it has been pointed out that it is not yet justifiable to assume that all triterpenes possess a common skeleton. A number of variants in the way of formulas have been successively considered during the past few years' accumulation of data to explain the origin of the series of hydrocarbons formed on dehydrogenation, but in some cases it has been necessary to assume definite rearrangements during their formation. The explanation of the origin of sapotalene has met with difficulty, although now, as Ruzicka points out, there is very strong evidence that it must arise from rings D and E of the picene system. After preliminary confusion as to its nature Ruzicka, Hofmann & Schellenberg have more recently succeeded in identifying the trimethylnaphthol frequently obtained as a dehydrogenation product as 1,2,5-trimethyl-6-hydroxynaphthalene (or hydroxyagathalene). This has caused them to discard earlier suggestions and to conclude that rings A and B of the pentacyclic terpenes are constructed in a manner identical with the corresponding portion of the molecule of most diterpenes such as agathenic dicarboxylic acid from which (and also tetracyclosqualene) 1,2,5-trimethylnaphthalene (agathalene) had first been obtained.

In their latest reports Ruzicka, Goldberg & Hofmann have proposed the formula LVII for oleanolic acid. This contains rings A and B in the form indicated by the degradation and dehydrogenation

results, together with ring C, constructed from a regular chain of 4 isoprene residues as they are to be found in diterpenes such as phytol, vitamin A, agathenic dicarboxylic acid, etc. Rings C to E, for which

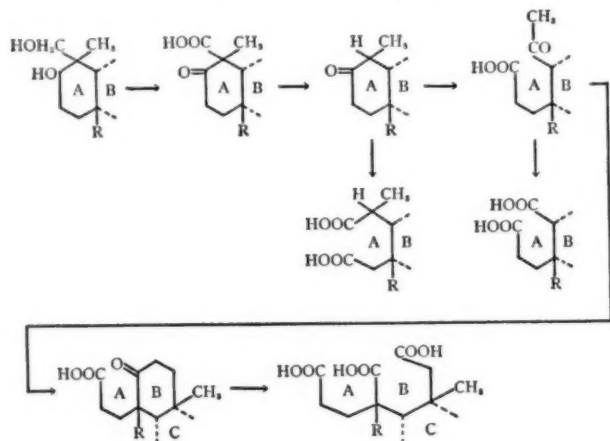


LVII

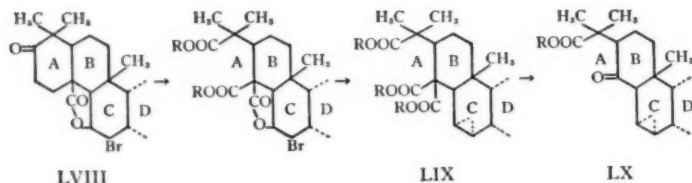
no final proof was available, are proposed as given in the picene structure, mostly on the basis of the interpretation of the origin of the dehydrogenation products. Among the latter which have been definitely characterized, 1,2,3,4-tetramethylbenzene can arise from ring A after rearrangement of a methyl group; 1,5,6-trimethyl-2-hydroxynaphthalene (1,2,5-trimethyl-6-hydroxynaphthalene) from rings A and B; 1,2,5,6-tetramethylnaphthalene also from rings A and B after rearrangement of a methyl group and cleavage of water; 2,7-dimethylnaphthalene and 1,2,7-trimethylnaphthalene (sapotalene) from rings D and E; and 1,7,8-trimethylphenanthrene from rings A to C. Finally, very recently Ruzicka and coworkers have identified among the dehydrogenation products of the amyrins 1,8-dimethyl-2-hydroxypicene which was definitely correlated with the often encountered 1,8-dimethylpicene that melts at 305°.

Kitasato in continuing his earlier work has more recently proposed a slightly different formula for oleanolic acid (LXI). With the exception of the position of the carboxyl group, this is in general agreement with that of Ruzicka and coworkers. As regards rings A and B it has been derived by the interpretation of products of the step by step oxidative degradation of hederagenin with simultaneous application of the so-called diterpene principle and the assumption of the close relationship of hederagenin and oleanolic acid. This degradation is represented by the partial formulas on page 466.

Of interest is the latest series of reactions reported by the Japanese worker. Starting with the monobromolactone of oleanonic acid (LVIII) and by oxidative degradation of ring A, the trimethyl ester of a tribasic acid (LIX) was finally obtained. This substance on



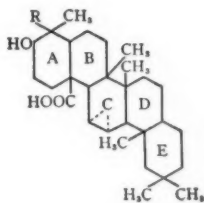
saponification gave, in turn, a keto acid ester (LX) in which two carboxyl groups were lost.



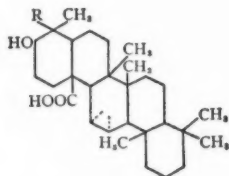
One of these was the original carboxyl group of oleanolic acid. The interpretation of this tribasic acid as a malonic acid derivative would necessarily place the original carboxyl group between rings A and B. Kitasato suggests therefore the formula LXI for oleanolic acid and hederagenin in which R is CH_3 and CH_2OH respectively, although the occasionally discussed symmetrical pentacyclosqualene type (LXII) was also considered by him. The latter, however, would be definitely excluded on the basis of the primary origin of 1,8-dimethylpicene.

Since in the case of certain derivatives, especially the so-called bromolactones, of these acid sapogenins the double bond has been found to participate in lactone formation with the carboxyl group,

a γ - δ orientation with respect to this carboxyl group has been assumed. This would now place it definitely in ring C. It appears to be quite certain that this double bond adjoins a CH_2 group so that ring C must be at least six-membered.



LXI



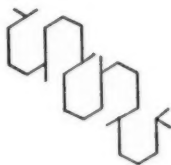
LXII

The masked character of the double bond in those sapogenins which cannot be hydrogenated also has led to the suggestion that instead of a double bond a cross bond may occur with production, therefore, of a sixth ring, which under certain influences might be ruptured with double bond formation. This appears to have been first considered by Wieland, Hartmann & Dietrich in their more recent work on quinovic acid. The latter does not respond either to hydrogenation or to the tetranitromethane test. The formulation $\text{C}_{30}\text{H}_{46}\text{O}_5$ of this substance, which is a monohydroxydibasic acid, would require either six rings, or five rings and a double bond. It has been similarly noted by Ruzicka and by Voss & Butter that glycerrhetic acid, $\text{C}_{30}\text{H}_{46}\text{O}_4$, likewise gives no test with tetranitromethane. However, again only in the past month Ruzicka & Prelog have found a probable reason for the failure of these substances to give the tetranitromethane test by showing that they possess double bonds masked by the influence of a carbonyl or carboxyl group. There appears, therefore, to be no further doubt that they belong to the pentacyclic group.

When rigidly considered, it may still appear to be a matter of conjecture in regard to the exact sizes and arrangements of rings D and E, although as previously discussed a very strong case has been made out for the picene structure. The question has been raised by Wieland, Hartmann & Dietrich in their discussion of quinovic acid by the suggestion that there is still a possibility that ring D may be five-membered as in the case of the sterols and that the picene hydrocarbons are produced by rearrangements.

The occurrence of another polybasic triterpenoid sapogenin (perhaps tetracyclic) has recently been reported by Jacobs & Isler in the case of senegenin. This substance, like quinovic acid, was found to be a dibasic acid, but to contain in addition a lactone group. On dehydrogenation it yielded apparently no naphthalene hydrocarbons, a minimal amount of 1,8-dimethylpicene, but in largest amount a hydrocarbon $C_{23}H_{22}$ with the properties of a polymethylchrysene. In the last respect it differed from quinovic acid which Wieland, Hartmann & Dietrich had found to give principally the picene hydrocarbon and small amounts of a different polymethylchrysene. The polymethylchrysene, $C_{23}H_{22}$, from senegenin, also proved to be identical with a hydrocarbon previously encountered by Ruzicka & Ehmann in very small amount as a dehydrogenation product of hedragenin. However, as Ruzicka and coworkers have shown, the course of the dehydrogenation of substances with the same ring system can be profoundly influenced by substituents, especially a hydroxyl group, and therefore different dehydrogenation products can preponderate in special cases.

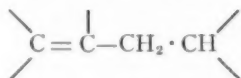
A novel interrelationship in the triterpene group has been reported during the past year by Beynon, Heilbron & Spring who noted the cyclization of a tetracyclic, diethenoid alcohol, basseol, from shea nut oil to pentacyclic β -amyrin, and is apparently the first example of such a conversion. This is of importance from a biological standpoint "in supplying the experimental realization of one stage in the natural evolution of a pentacyclic triterpene from presumably an acyclic structure of the squalene type (LXIII) by means of stepwise cyclization."



LXIII

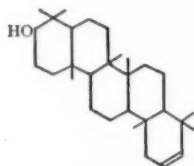
Attempts to use surface film measurements in approaching these complicated problems have been reported by Askew. Measurements of surface pressures and surface potentials have been made and interpreted in connection with proposed structures.

Also of interest has been a series of papers by Spring, mostly with Vickerstaff, on oxidative transformations with α -amyrin (α -amyrenol), and which appeared before the very recent work of Ruzicka & Schellenberg on the conversion of oleanolic acid to β -amyrin. Among other very special points a necessary confirmation by direct chemical means was obtained of the unsaturated nature of α -amyrin previously inferred from the color reactions and analyses. This unsaturated center has been defined as



located in Ring C. Although they also favored for α -amyrin the above hydrocypicene formula of the symmetrical type derived by a series of cyclizations of squalene, such a formula appears now excluded by the dehydrogenation experiments of Ruzicka & Hofmann and Ruzicka, Schellenberg & Goldberg.

Very recently the possibility has been considered with necessary reservations by Ruzicka, Furter, Pieth & Schellenberg that lupeol, $\text{C}_{30}\text{H}_{50}\text{O}$, may be constructed on a symmetrical pentacyclic squalene framework. On dehydrogenation it was found to yield both hydroxyagathalene and agathalene itself, but no sapotalene. In the cases of all other pentacyclic triterpenes from which hydroxyagathalene has been obtained, agathalene itself has not been simultaneously detected as a dehydrogenation product. In such cases sapotalene has been the only trimethyl naphthalene which could be isolated. Accordingly (LXIV) has been discussed by them as a possible structure for lupeol.



LXIV

Finally, in this country Noller and Drake and their coworkers have made contributions in the triterpene group. The former isolated a sapogenin, echinocystic acid, from the roots of *Echinocystis fabacea*, which proved to be a dihydroxy acid, $\text{C}_{30}\text{H}_{48}\text{O}_4$, and on dehydrogenation yielded the usual hydrocarbons. Drake and co-

workers have studied the long-known substances occurring in cork, cerin and friedelin—the former a hydroxyketone and the latter a ketone. Their triterpenoid nature was shown by dehydrogenation studies.

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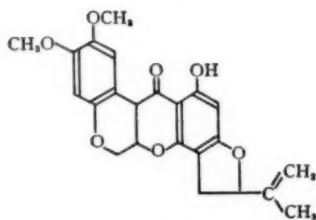
The organic insecticides discussed in this review are only such compounds as have plant origin. In this category are included only the compounds of the rotenone group found principally in derris and cubé, the pyrethrins, quassin, and nicotine and related alkaloids.

COMPOUNDS OF THE ROTENONE GROUP

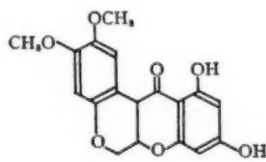
Sumatrol.—Cahn & Boam (1) isolated a new compound of the empirical formula $C_{23}H_{22}O_7$, isomeric with tephrosin and toxicarol, from a species of derris, probably *Derris malaccensis*. Preliminary tests indicated that the compound had insecticidal properties, and certain chemical reactions showed the similarity of the new compound to toxicarol. Robertson & Rushy (2) took up the study of sumatrol and confirmed the empirical formula of Cahn & Boam. The presence of a ketone group, a phenolic hydroxyl group, and two methoxyl groups was established. It was found that, like rotenone, deguelin, and toxicarol, sumatrol yielded a dehydro derivative on oxidation. It could not be dehydrated by mineral acids, differing in this respect from compounds of the tephrosin type. By scission with alkali the dehydro derivative yielded sumatrollic acid, a reaction that was analogous to the formation of derrisic acid from rotenone. Sumatrol yielded a mixture of the dihydro and the diphenolic tetrahydro derivatives on hydrogenation. Its behavior in this respect was analogous to that of rotenone and served to differentiate it from deguelin, toxicarol, and tephrosin. Sumatrol would therefore contain the isopropenyldihydrofuran nucleus characteristic of rotenone. On the basis of the reactions described and the resistance of its phenolic hydroxyl group to methylation, formula I has been assigned to sumatrol.

Toxicarol.—From the work of Clark and of Butenandt done between 1930 and 1933 toxicarol ($C_{23}H_{22}O_7$) had been shown to possess a chromanochromanone nucleus of the rotenone type, which could be oxidized to the dehydro form. The dehydro derivative could be hydrolyzed to toxicarolic acid and this in turn degraded to derric and dehydronetic (XII) acids. Hence the chromano residue is identical with that present in rotenone.

* Received January 24, 1938.



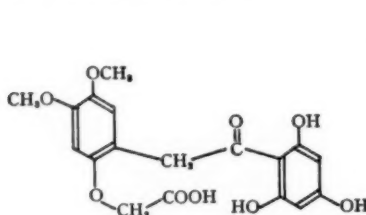
I. Sumatrol



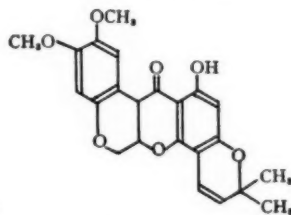
II. Apotoxicarol

Toxicarol contains a phenolic hydroxyl group. On treatment with alkali it yields apotoxicarol ($C_{18}H_{16}O_7$) with loss of five carbon and six hydrogen atoms, a reaction that is not characteristic of rotenone.

Apotoxicarol undergoes the reactions characteristic of the chromanochromanone residue and has been assigned formula II. Toxicarol does not show the characteristic behavior of rotenone on hydrogenation and hence contains no dihydrofuran ring. To determine the position of the hydroxyl group and the nature of the ring that is present, and which is removed by alkali treatment, Robertson & Heyes (3) prepared an acid of formula III by synthesis and found



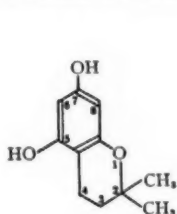
III. Apotoxicarolic acid



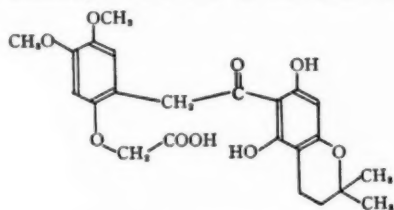
IV. Toxicarol

it to be identical with apotoxicarolic acid of the same formula obtained from apotoxicarol. Thus, the positions of the phenolic hydroxyl groups present in apotoxicarol were located. Since in compounds of the type of apotoxicarol a chelate system obtains and the hydroxyl in the ortho position to the carbonyl is difficult to methylate, the original phenolic hydroxyl is probably in this position. Toxicarol and also deguelin yielded acetone on treatment with alkali under the proper conditions. This indicates the presence of the grouping $(CH_3)_2C$ and supports the theory that toxicarol also contains the 2,2-dimethyl- Δ^3 -chromene residue characteristic of deguelin. On the basis of this evidence, and by analogy, toxicarol was tentatively assigned

formula IV. Substantiation of the nature of the chromene system just postulated was furnished in a subsequent article by Bridge, Heyes & Robertson (4) based on the following results: Dihydrotoxicarolic acid, which had been previously described by Clark (5), yielded by scission with alkali a crystalline dihydric phenol, $C_{11}H_{14}O_3$, which did not contain a carbonyl group and which yielded a diacetate. On the basis of a 2,2-dimethyl- Δ^3 -chromene hypothesis and the fact that apotoxicarol had been shown to contain a phloroglucinol residue, the compound was considered likely to be 5,7-dihydroxy-2,2-dimethyl-chromane, which corresponds to formula V. This compound was synthesized and its structure definitely established. It was found to be

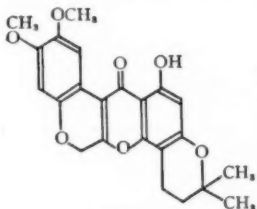


V

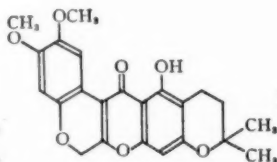


VI. Dihydrotoxicarolic acid

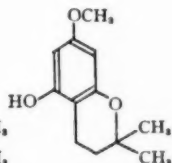
identical with the phenol obtained from dihydrotoxicarolic acid. Dihydrotoxicarolic acid could then be represented only by the formula VI. Hence, dehydrodihydrotoxicarol must be represented by one of the alternate structures VII or VIII, and corresponding formulae would apply to toxicarol itself.



VII



VIII



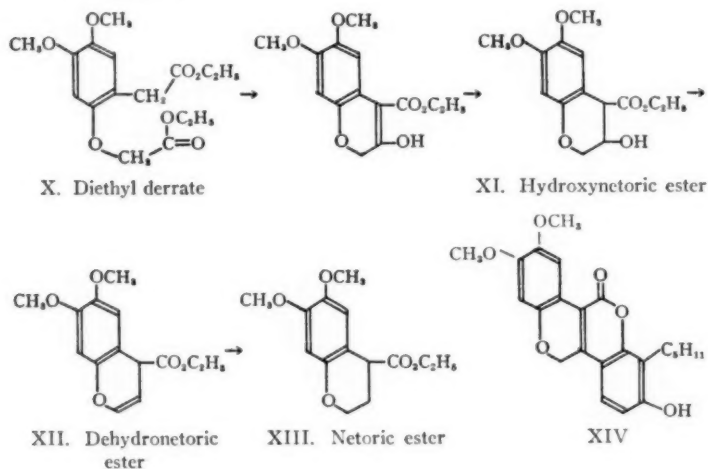
IX

By the following procedure a choice in favor of formula VII was made by George & Robertson (6). Dehydrodihydrotoxicarol was methylated on the phenolic hydroxyl and the methyl ether subjected to scission with alkali with the formation of a monomethyldihydrotoxicarolic acid. Fission of this acid with concentrated alkali yielded

derric acid and 5-hydroxy-7-methoxy-2,2-dimethylchromane of formula IX. The structure of this compound was established by comparison with the synthetic compound in which the orientation of the methoxyl group had been confirmed.

These facts definitely establish the formula of dehydrodihydrotoxicarol, and hence the formula IV for toxicarol itself. The probable mode of formation of acetone from toxicarol and compounds with the 2,2-dimethyl- Δ^3 -chromene grouping is discussed by George & Robertson.

The esters of netoric, hydroxynetoric, and dehydronetoric acids, degradation products of rotenol (7, 8) and toxicarol (5), have been synthesized by Robertson & Rusby (9) from diethyl derrate (X) by the following steps:



The esters on saponification yielded the free acids identical with the natural ones. The natural netoric acid was stated by Smith & LaForge (7) and by Clark (5) to melt at 130° and to form a hydrate melting at 91° . Takei (10) and Butenandt & McCartney (11) considered the two forms to be dimorphic. The natural as well as the synthetic netoric acids, according to Robertson & Rusby, formed a hydrate melting at 90° , and the anhydrous acid melted at 132° .

Chromenocoumarins.—Robertson and his coworkers (12) have investigated a series of chromenocoumarins of the general type XIV

with the object of making a comparative study of their physiological action. A number of these have been prepared as well as other types. Robertson & Rusby (13) prepared tetrahydrotubaic aldehyde from tubanol by reaction with zinc cyanide, hydrogen cyanide, and hydrochloric acid. It yielded a diacetate that on oxidation was converted into the diacetate of tetrahydrotubaic acid. Tetrahydrotubanol, which had already been synthesized by Haller (14), has been synthesized by Robertson & Subramaniam (15) in a more convenient way by the following steps: Application of the Fries reaction to the isovalerate of 7-hydroxy-4-methylcoumarin gave 7-hydroxy-8-isovaleryl-4-methylcoumarin, which by scission furnished 2,6-dihydroxyisovalerophenone, which was reduced to tetrahydrotubanol. The method would not establish the orientation of the isoamyl group.

For an understanding of the fundamentals of rotenone chemistry the reader is referred to reviews on the subject by LaForge, Haller & Smith (16), Butenandt & McCartney (11), and King (17).

Toxicity and physiological action.—The literature under this heading, especially with regard to insecticidal action, is so extensive that a comprehensive review would greatly exceed the scope of this article. Such reviews have been prepared by Roark on *Derris* (18), *Tephrosia* (19), and *Lonchocarpus* (20). The more recent literature has been reviewed by Chevalier (21).

The insecticidal activity of derris and cubé is by no means proportional to the rotenone content but may be due to the content of the optically active forms of the known constituents that have so far been isolated only in the inactive forms. The rotenone-containing plants are toxic to nearly all insect forms, although a few are immune to their action. Some attack the growing plant and some the dried stored roots. Fink & Haller (22) found that *l*-dihydrodeguelin was ten times as toxic to mosquito larvae as inactive dihydrodeguelin. They inferred from this that optically active deguelin, which has not yet been obtained, would be more toxic than the inactive form, and that the toxicity of the non-rotenone constituents of derris might be due in part to the presence of active deguelin. In this connection Cahn (23) calls attention to the possible existence in derris of several geometric isomeric forms of deguelin which may have a bearing on its physiological action.

Gersdorff (24) tested the toxicity of a number of rotenone derivatives on goldfish. On the basis of rotenone as unity, the order of toxicity was found to be dihydrorotenone 1.4, acetyldihydrorote-

none 0.81, acetylrotenone 0.55, dihydrorotenolone 0.15, rotenolone 0.097, acetyldihhydrorotenolone 0.082, and acetylrotenolone 0.055.

Much work has been done on the chemical and biological evaluation of rotenone-bearing plants. Space limitations permit reference only to articles by Jones & Smith (25), who found that the toxicity of derris to house flies could be expressed by the toxicity of the rotenone content plus one-half of the carbon tetrachloride or benzene extractives other than rotenone, and by Tattersfield & Martin (26), who concluded that the content of derivatives capable of forming dehydro compounds gave the best correlation with toxicity to *Aphis rumicis* L.

Because of the employment of preparations of rotenone-bearing plants as arrow poisons, there was apprehension as to their possible serious effect on man and animals. Pharmacological studies on this subject have been made by Buckingham (27), Haag (28), Ambrose & Haag (29), and Mathews & Lightbody (30). The results obtained by these authors on dogs, cats, rats, chickens, and other animals indicate that there is no danger from the ingestion of such amounts of rotenone or derris as would ordinarily be encountered in food products to which preparations of these substances had been applied as insecticides. For instance, it was concluded that rotenone was only 1/100th as toxic to rats as lead arsenate. On the other hand, when rotenone or derris preparations are injected intravenously, comparatively small doses cause respiratory disturbances followed by paralysis, cardiac symptoms, lowering of the blood pressure, and finally death. The cold-blooded animals are all more sensitive than the warm-blooded animals to derris and rotenone whether applied by ingestion or contact.

THE PYRETHRINS

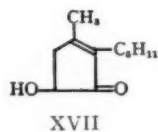
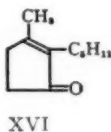
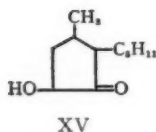
Chemistry.—Ripert (31) has described the isolation of pyrethrins I and II by physical means. The method is based on the observation that pyrethrin II is much less soluble in pentane than pyrethrin I and may be obtained pure by chilling a solution of the mixed pyrethrins in this solvent to -50° . By repeated precipitation pyrethrin II of 98 per cent purity was obtained. Pyrethrin I of 96 per cent purity was obtained from the pentane solution after the remaining pyrethrin II had been removed by chilling to -120° . No analytical data are presented, and it is not clear how the author obtained the pure mixture of the two pyrethrins on which the separation was made. The

report cannot but leave doubt as to the quality of the materials described.

LaForge & Haller (32) have described a method for the partial separation of the two pyrethrins. Petroleum-ether extractives of pyrethrin flowers obtained commercially were freed from fats, waxes, and gross impurities by treatment with acetic acid containing 10 per cent of water. The insoluble material was removed by filtration, and a concentrate containing 60 to 65 per cent of total pyrethrins was obtained from the acetic acid solution. Free fatty acids were removed from the concentrate by treatment with aqueous sodium carbonate in aniline solution. The aniline was removed with hydrochloric acid and the concentrate of about 70 per cent total pyrethrins separated by differential solubility between petroleum ether and 90 per cent aqueous acetic acid. Pyrethrin I, together with most of the extraneous material, tends to concentrate in the acid. Repetition of the process gave concentrates of 40 to 55 per cent pyrethrin I with 12 to 15 per cent pyrethrin II, and 80 to 85 per cent pyrethrin II with 3 to 6 per cent pyrethrin I. By molecular fractional distillation chemically pure pyrethrin II could be obtained from the pyrethrin-II concentrate, but the product was in some manner altered by distillation and it did not yield a crystalline semicarbazone.

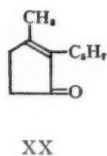
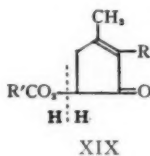
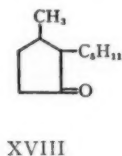
The semicarbazones of the pyrethrins and of pyrethrolone have been investigated by Haller & LaForge (33). Pyrethrin-II semicarbazone was obtained as a pure chemical individual from a pyrethrin-II concentrate, but the semicarbazone of pyrethrin I could not be prepared in a pure state. Repeated analysis of pyrethrolone semicarbazone showed that its formula, $C_{12}H_{17}O_2N_3$, contained two hydrogen atoms less than the formula proposed by Staudinger & Ruzicka. Tetrahydropyrethrolone semicarbazone also was shown to have the empirical formula $C_{12}H_{21}O_2N_3$ instead of $C_{12}H_{23}O_2N_3$, and pyrethrin-II semicarbazone likewise contained two hydrogen atoms less than the previously accepted formula.

LaForge & Haller (34) prepared pyrethrolone and tetrahydropyrethrolone from their semicarbazones and showed that their respective empirical formulae were $C_{11}H_{14}O_2$ and $C_{11}H_{18}O_2$. Since both these formulae contain two atoms of hydrogen less than were previously assigned to these compounds, it was concluded that an error existed in the original structural formulae. Formula XV for tetrahydropyrethrolone, as proposed by Staudinger & Ruzicka, contains a cyclopentanolone nucleus. It was found that the hydroxyl



group in tetrahydropyrethrolone could be substituted by chlorine and that the chlorine could be replaced by hydrogen. The resulting compound was shown by analysis and by its properties and those of its derivatives to be identical with dihydrojasnone of the known structure XVI. Tetrahydropyrethrolone therefore contains the cyclopentenolone nucleus and should be represented by formula XVII instead of XV. A corresponding formula applies to pyrethrolone, with the nature of the double bond system in the side chain still uncertain. Complete reduction of pyrethrolone or of its acetate results in the formation of hexahydropyrethronone of formula XVIII.

Since the acetate of pyrethrolone is cleaved on hydrogenation with the formation of hexahydro- and tetrahydropyrethronone, the behavior on hydrogenation of the pyrethrins themselves, which are also esters of pyrethrolone, was studied by Haller & LaForge (35). A concentrate containing about 80 per cent of pyrethrin II in ethanol solution yielded a mixture of tetrahydro- and hexahydropyrethronones (formulas XVI and XVIII) together with the monomethyl ester of chrysanthemum dicarboxylic acid. A concentrate containing about 54 per cent of pyrethrin I and 11 per cent of pyrethrin II by the same process yielded the same pyrethronone derivatives and a mixture of dihydrochrysanthemum monocarboxylic acid and the monomethyl ester of chrysanthemum dicarboxylic acid. The cleavage of the pyrethrins by hydrogenation therefore proceeded as represented by XIX, where R' represents the radicals of the acids characteristic of each of the pyrethrins. The reaction is analogous to the cleavage of acetylpyrethrolone by hydrogenation. LaForge & Haller (36) observed that the semicarbazone of tetrahydropyrethronone corresponding to formula XVI is easily soluble in dilute hydrochloric acid, while that



of hexahydropyrethron corresponding to formula XVIII is not, and that one could be separated from the other by taking advantage of this property.

Since it had not been possible to obtain the semicarbazone of pyrethrin I in pure condition by recrystallization, the best obtainable preparations of this derivative were examined by LaForge & Haller (37). The semicarbazone was hydrolyzed, yielding pyrethrolone semicarbazone as the only neutral reaction product. The acid fraction proved to be a mixture containing, besides the expected chrysanthemum monocarboxylic acid, chrysanthemum dicarboxylic acid and an unsaturated fatty acid. The latter was suspected to be a mixture. Preparations of pyrethrin-I semicarbazone therefore consist of mixtures containing the semicarbazone of pyrethrin II and the semicarbazone of esters of pyrethrolone with one or more fatty acids. The investigation was continued by Acree & LaForge (38), who found that the fatty acids present combined with pyrethrolone in preparations of pyrethrin-I semicarbazone consisted of a mixture of palmitic and levulinic acids, which were separated and identified.

Tetrahydropyrethrolone had been reported by Staudinger & Ruzicka to show an optical rotation of -13.0° . This derivative had been found by Haller & LaForge to rotate to the same degree but in the opposite direction. Since in both cases the pyrethrolone had been obtained from tetrahydropyrethrolone semicarbazones originating from mixtures of the semicarbazones of pyrethrins I and II, there was the possibility that in one of the pyrethrins the *d*-form of pyrethrolone was present, and in the other the *l*-form. To decide this question Haller & LaForge (39) prepared pyrethrolone from the semicarbazones of pyrethrins I and II. The pyrethrolone obtained from pyrethrin I was found to rotate $+14.8^\circ$, that from pyrethrin II, $+17.7^\circ$. The difference was attributed to partial racemization. It was concluded that both pyrethrins contain the same optical form of pyrethrolone. The values are markedly different from that (-6.0°) reported for pyrethrolone by Staudinger & Ruzicka.

Pyrethrolone obtained from both sources was esterified with chrysanthemum dicarboxylic acid monomethyl ester, but the synthetic pyrethrin II did not yield the characteristic semicarbazone of pyrethrin II. However, when tetrahydropyrethrolone from both sources was esterified with the chrysanthemum dicarboxylic acid monomethyl ester, the semicarbazone of tetrahydropyrethrin II was obtained.

LaForge & Haller (40) have investigated the nature of the side chain of pyrethrolone to determine the relative positions of the two double bonds. Staudinger & Ruzicka had deduced the presence of an allene system. Because such a system had never been observed in nature, doubt as to its existence in the pyrethrins had been expressed by chemists, among them one of the authors of the original communications. The evidence did not seem to have completely excluded the presence of a conjugated system. The results of LaForge & Haller (40) seem to exclude the latter consideration but do not furnish proof of an allene system. The experiments were made largely with pyrethronone of formula XX obtained by reduction of pyrethrolone with aluminum amalgam. Pyrethronone still retained the original unsaturated system in the side chain and yielded tetrahydropyrethronone (dihydrojasmonone) on hydrogenation. It did not undergo the Diels-Alder reaction with maleic anhydride or with α -naphthoquinone. It yielded acetaldehyde on ozonization. Its most characteristic behavior was seen in its reaction with bromine. In organic solvents it reacted with one mol of bromine with formation of a monobromo derivative and liberation of one mol of hydrobromic acid.

The reaction was therefore equivalent to a substitution. A second mol of bromine reacted with the monobromo compound in the same manner as did the first, with formation of a dibromo compound and liberation of another mol of hydrobromic acid. Both the monobromo and the dibromo derivatives furnished the original pyrethronone on reduction with zinc. All these reactions, including the stability of the unsaturated system toward aluminum amalgam, the reagent employed in its preparation, are against the hypothesis of a conjugated system. The formation of acetaldehyde serves to locate the position of one double bond in the side chain, but the other reactions cited do not fix the position of the other double bond. The presence of the allene system in pyrethronone can be considered as likely. Pyrethrolone was found to behave with respect to the reactions described in the same manner as did pyrethronone.

An observation made by Ripert (41) regarding the extraction of the pyrethrins from pyrethrum flowers with hydrocarbon solvents is of special importance. The author demonstrated that when dried flowers were percolated with petroleum ether a considerable proportion of the toxic principles was not extracted but could be removed by a subsequent extraction with chloroform, which solvent is capable of extracting the total pyrethrin content. In the process of drying,

part of the pyrethrins becomes surrounded with an insoluble film formed by oxidation of a natural constituent of the flowers and is protected from the solvent action of hydrocarbons. This assumption is supported by the results of experiments.

Toxicity and physiological action.—As in the case of rotenone and derris, the literature on the physiological action of the pyrethrins is very extensive. Reports on this subject have been published by Bacq (42), Buchmann (43), Campbell (44), Gnädinger & Corl (45), Hartzell & Wilcoxon (46), and Tattersfield (47). It seems to be established that both pyrethrins are practically non-toxic to warm-blooded animals, but a wide difference of opinion still exists regarding the relative toxicity of pyrethrins I and II toward insects.

Gnädinger has published the second edition of his text and reference book *Pyrethrum Flowers*, in which the literature to 1936 is reviewed. He also describes the various methods of analyses in use, both chemical and biological.

Gaudin (48) described tests made on a large number of species of animals, including some insects. The author confirmed the prevailing opinion that the pyrethrins are practically non-toxic when ingested. He found them to be toxic to all animals when introduced into the circulation, and their effect involved the nervous system. The striking observation was made that crustaceans were 1,000 to 10,000 times as sensitive to the pyrethrins as any other class of animals. The relative toxicities of pyrethrin I and pyrethrin II were found to vary widely for different species, some proving more sensitive to pyrethrin I and some to pyrethrin II. Too much value should not be attached to the last-mentioned results, since the pyrethrins employed may have been incompletely separated. Gaudin's monograph contains a bibliography of previous work on the toxicity of the pyrethrins.

QUASSIN

Clark (49) has begun a study of the bitter and physiologically active constituents of quassia wood, which for one hundred years has been known to contain the crystalline principle quassin. A new method for obtaining the active principle has been devised which involves its adsorption on active carbon and subsequent recovery by elution of the adsorbed material with chloroform. Various formulae have been proposed for quassin since its discovery. Clark describes the separation of the crystallate into two isomers, for one of which the original name has been retained and the other has been named

"neoquassin." Both have the empirical formula $C_{22}H_{30}O_6$, and both contain two methoxyl groups and one active hydrogen atom. Quassin melts at 206° , neoquassin at 226° . Both lose one methoxyl group on mild treatment with hydrochloric acid. On more drastic treatment with the same reagent quassin yields quassinol, $C_{20}H_{24}O$, with properties resembling those of an acid. Quassin forms an anhydro and a dehydro compound on treatment with acetic anhydride. On treatment with chromic acid a 50 per cent yield of an isomer, isoquassin, is obtained.

Neoquassin (50) resembles quassin closely in most of its reactions. Boiling with 5 per cent ethanolic potassium hydroxide has no effect on neoquassin but changes quassin in such a way as to permit its removal from a mixture of the two, and the method is used for purification of the former. Neoquassin, on mild treatment with hydrochloric acid, yields the same semidemethoxyquassin as does quassin, but no quassinol is obtained by more drastic treatment with this reagent. Chromic acid treatment of neoquassin leads to the formation, in 50 per cent yield, of isoquassin. Neoquassin can be converted by acetic anhydride into anhydroquassin, but no dehydroquassin is formed. Isoquassin under the conditions described for the formation of anhydroquassin gives dehydroquassin.

NICOTINE AND RELATED COMPOUNDS

Two bibliographies on nicotine have recently been issued by the United States Department of Agriculture. The first of these, by Busbey & McIndoo (51), covers the chemistry of nicotine, with 851 references, while the second, by McIndoo, Roark & Busbey (52), covers the insecticidal uses of nicotine and tobacco, and has 2,497 references.

Analytical methods.—A valuable method for the estimation of small amounts of nicotine by a colorimetric procedure has been applied to tobacco by Barta & Marschek (53). The method depends upon the formation of a red color with cyanogen bromide and β -naphthylamine. A sample of tobacco weighing 10–50 mg. is shaken with 1 cc. of cyanogen bromide solution and 10 cc. of a 0.1 per cent solution of β -naphthylamine in 50 per cent alcohol; after 12 hours the color developed is compared with a standard in a colorimeter. This reaction, formerly applied to pyridine and applicable to any pyridine derivative, is capable of estimating nicotine in dilutions of 1 to 1,000,000.

Spies (54) has worked out a micromethod for nicotine based on

the silicotungstic acid precipitation. Goodhue (55) determines small amounts of nicotine by turbidimetric titration involving the silicotungstate, using a photoelectric cell. Chamberlain & Clark (56) modify the Pfyl & Schmitt method (57) by weighing the nicotine dipicrate instead of titrating it. Rytikov (58) describes a rapid micromethod for tobacco wherein the toluene extract, obtained after treatment of the tobacco with calcium oxide, water, and gypsum, is titrated with acid. The distribution coefficients between aqueous nicotine solutions and a number of organic solvents were determined by Kolosovskii & Kulikov (59). Katz (60) gives two reactions for differentiating nicotine and anabasine: (a) anabasine is quantitatively precipitated by fluosilicic acid, while nicotine remains clear; (b) nicotine in ether solution is precipitated with ethereal iodine solution (Roussin reagent) as the periodide, while anabasine is not. Gold chloride is also reported by Zerbey, Orinick & Willard (61) to furnish a differentiation between these two alkaloids.

Toxicity and physiological action.—Interest in the pharmacological effects of nicotine continues unabated. Staemmler (62) reports that repeated subcutaneous injections of nicotine, as either the free base or the picrate, did not produce detectable injury to the genital glands of rats. No changes in secondary sexual characteristics were noted. Morra (63) administered daily injections of aqueous nicotine solutions to pregnant rabbits, with the result that delivery of the fetus occurred eight to thirteen days earlier than in the controls. The fetuses contained nicotine. Kobayashi (64) found that acute doses of nicotine in rabbits caused marked hyperglycemia. In cases of chronic poisoning by small daily doses of nicotine tartrate, there was first an increase in blood sugar and then a gradual decrease, culminating in hypoglycemia. He also investigated the glycogen content of the liver and the adrenaline content of the suprarenals following nicotine poisoning. Wilson & De Eds (65) investigated the effect of nicotine on the estrus cycle of rats. A diet containing 0.025 per cent of nicotine caused a prolonged and irregular cycle, and a diet of 0.05 per cent stopped the cycle completely. The evidence indicates that the alteration of the cycle is due to decreased food intake, not to direct poisoning. When nicotine is introduced into the pericardial cavity of the dog (66), there is slow absorption, and unusually large doses can be thus tolerated. The cardiac contraction rate of isolated heart preparations from the army worm and the cockroach when perfused with nicotine was investigated (67). Stimulation followed by partial or

complete depression, depending on the concentration, resulted. The order of toxicity of three related compounds, determined by penetration experiments on the cockroach, was found to be nicotine > piperidine > pyridine (68). Ellisor (69) investigated the apparent differences in toxicity of free nicotine and its salts to goldfish. The rates of penetration were different, being less with increase of ionization, but at death the body contained 0.034 mg. per gm. irrespective of which type of compound was used. Leont'ev (70), on the other hand, as a result of studies on the isolated frog heart and on the spinal reflex of the frog, concluded that free nicotine was more toxic than a casein-nicotine complex.

An important contribution to the treatment of acute nicotine poisoning was made by Franke & Thomas (71). They found, on trials with dogs, that artificial respiration was uniformly successful if it was begun before the circulation failed and was continued until muscular paralysis disappeared. Prolonged artificial respiration and, when the heart has stopped, intracardiac injection of epinephrine are recommended for trial in cases of acute nicotine poisoning. Certain drugs, including nicotine, cause an increased secretion of the prostate gland (72). The distribution of nicotine in the blood between plasma and cells depends upon the concentration of the nicotine. At 0.3 mg. per 10 cc. it is found only in the cells, at 0.6 mg. per 10 cc. it is found moderately strong in both, while at 0.9 mg. per 10 cc. the plasma gives the more intense reaction (73).

Tobacco smoke.—The subject of tobacco smoke has continued to receive considerable attention. Bradford *et al.* (74) studied the relation of acidic and basic constituents of the smoke, showing that it depends on the composition of the tobacco and its physical treatment. Derr and associates (75), in a study of the absorptive properties of various media, including activated alumina, found that the most efficient, as well as the simplest, absorbent for nicotine is an ordinary cigaret, which is placed in a holder behind the burning cigaret. One such filter is usable for 35 to 40 cigarets. Wenusch (76) finds that the size of the cigaret is without material influence on the amount of nicotine in the main smoke stream. He determined the nature and amount of both liquid and solid constituents of smoke. Preiss (77) uses titration methods for determining ammonia and pyridine bases in smoke, and draws conclusions in regard to the decomposition of the various tobacco constituents.

New insecticidal preparations and their application.—In the field

of practical insecticide application the endeavor in the last few years has been to develop "fixed" nicotine preparations, that is, insoluble types. Nicotine tannate, an early such product, has been tested for corn-borer control by Batchelder, Questel & Turner (78). Nicotine bentonite (79) is now coming into use for codling moth control on apples. Nicotine peat (80) is still in the experimental stage, requiring development of a suitable sticker. Nicotine humate (81), nicotine sulfite (82), nicotine thiocyanate (83), and nicotine in refined petroleum distillate (84), are other recently described combinations. A method for determining nicotine on apples sprayed with nicotine bentonite, by base-exchange with brucine, has been worked out by Markwood (85).

Related alkaloids.—Alkaloids related to nicotine and species of *Nicotiana* other than *tabacum* have been intensively investigated. Späth & Keszler (86) report the occurrence of *d,l*-nornicotine, *d,l*-anatabine, and *l*-anabasine in tobacco. Smith (87), after having found anabasine in *N. glauca*, investigated *N. sylvestris* and found it to contain *l*-nornicotine, which was present to the extent of about 95 per cent of the total alkaloids, the remainder being *l*-nicotine (88). An important background to the study of the various *Nicotiana* species resides in the work of Shmuk & Khmura (89), who investigated the alkaloidal content of many species, as well as numerous hybrids. They divided the alkaloids into volatile and non-volatile groups, without achieving positive identification. Khmura (90) points out that *N. glauca* may become of commercial importance because of both its anabasine content, 0.74 per cent, and its content of acids (citric 7.16 per cent and malic 7.90 per cent). It is known that attempts have been made before to base a tobacco industry on recovery of the organic acids.

Norkina and coworkers (91) found that partial racemization of anabasine occurs in its isolation by some methods, which fact, no doubt, accounts for the varying values of optical rotation found in the literature. When anabasine is obtained by direct formation of a nitroso derivative, $[\alpha]_D = -69.0^\circ$, but when it is recovered from a dichloroethane extract of the plant by formation of the same compound, $[\alpha]_D = -81.0^\circ$. The symptoms of anabasine poisoning resemble those of nicotine, according to Sarguine (92). Investigations of Richardson and coworkers (93) with the nicotines, nornicotines, and anabasine against the bean aphid (*Aphis rumicis* L.) show the following order of toxicity: anabasine > *l*- β -nicotine = *d,l*- β -nor-

nicotine $> d,l\text{-}\beta\text{-nicotine} > d,l\text{-}\alpha\text{-nicotine} = d,l\text{-}\alpha\text{-nornicotine}$. The distribution of nicotine in tobacco-tomato grafts reveals that nicotine migrates from tobacco stock into the tomato scion but not from tobacco scion into tomato stock (94).

The successful use of nicotinic acid in the treatment of canine black tongue (95) brings new interest to the subject of pellagra control. Various cyclic esters of nicotinic acid have been prepared (96).

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BUREAU OF ENTOMOLOGY AND PLANT QUARANTINE
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THE CHEMISTRY OF BACTERIA*

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This review covers only studies of a well-defined chemical character. This restriction excludes such work as the fermentation of various carbon compounds (frequently called "biochemical properties of bacteria," and usually of a qualitative character) where the products other than gas and acid are not stated. In general, abstracts of papers read at scientific meetings are not mentioned, because of limitations of space and because of the difficulty of evaluating such reports from the brief published abstracts. Also, the more important papers given at such meetings usually appear later in more finished form in the journals and can be covered adequately in later reviews.

NUTRIENT REQUIREMENTS OF BACTERIA

Growth substances.—Lwoff & Lwoff (1, 2) have shown that factor "V," which is necessary for the growth of certain haemophilic organisms, e.g., *Haemophilus parainfluenzae*, can be completely replaced by small quantities of either Euler's cozymase (coenzyme I) or Warburg's coenzyme (coenzyme II). Different components of the coenzyme molecules, such as adenylic acid, nicotinic acid or its amide, proved unable to act as growth factor "V." The limit of activity of pure pyridine nucleotide triphosphate (coenzyme II) was about 1 part in 600,000,000 parts of medium (0.0017 $\mu\text{g. per cc.}$).

Some species of *Haemophilus* (*H. canis* and *H. ducreyi*) can synthesize the pyridine nucleotide but require small amounts (0.03 $\mu\text{g. per cc.}$) of hematin (factor X). Still another species (*H. influenza*) requires both hematin and nucleotide (0.001 $\mu\text{g. per cc.}$ of the latter) (3).

Knight (4) has reported that the basic factor necessary for the growth of *Staphylococcus aureus* in acid-hydrolyzed gelatin can be replaced by mixtures of vitamin B₁ and nicotinic acid or its amide. 0.02 $\mu\text{g.}$ of vitamin B₁ plus 2.0 $\mu\text{g.}$ nicotinic acid per 10 cc. of medium gave abundant growth. In the presence of an excess of the other factor, vitamin-B₁ potency could be detected in concentrations of $4.0 \times 10^{-10}M$ (0.0012 $\mu\text{g. per 10 cc.}$) and that of nicotinamide in concentrations of $2.6 \times 10^{-8}M$ (0.032 $\mu\text{g. per 10 cc.}$). Nicotinic

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acid was identified spectrographically by Holiday (5) in highly potent fractions containing the *Staphylococcus* factor. Studies on the specificity of vitamin B₁ (6) showed that a mixture of the pyrimidine plus the thiazole, both substituted as in vitamin B₁, could replace the latter, while a differently substituted pyrimidine or thiazole was inadequate. Thiochrome could also replace the vitamin. Richardson (7) showed that uracil was required in addition to vitamin B₁ and nicotinic acid for anaërobic growth of certain strains of *Staphylococci*. It is now possible to grow *Staph. aureus* either aërobically or anaërobically on a completely synthetic medium (8, 9).

Continued study of the nutritional requirements of the diphtheria bacillus by Mueller has shown that pimelic acid (10, 11, 12), nicotinic acid (13, 14), and β -alanine (15) are required by that organism for growth on a modified hydrolyzed casein medium. Pimelic acid (11) and nicotinic acid (14) were isolated from concentrates exhibiting the same effect on growth as these substances; the presence of β -alanine in a third concentrate seems reasonably certain (15). The optimum concentration of pimelic acid was 0.25 μ g. per 10 cc.; its effect was detectable in a concentration of 0.05 μ g. per 10 cc. The optimum concentration of nicotinic acid was 10 μ g. per 10 cc.; it was easily detectable in a concentration of 0.1 μ g. per 10 cc. β -Alanine gave a maximum effect in amounts as low as 10 μ g. per 10 cc. of medium; carnosine could replace it in somewhat higher concentration. Addition of these three substances in optimum quantities to the basal medium yielded two-thirds as much growth as when extract of liver tissue was added.

Wood, Tatum & Peterson (16) and Fromageot & Piret (17) described properties of an unidentified substance obtained from corn, yeast, and other plant materials which is indispensable for all species of *Propionibacterium* tested; its presence in the medium allowed utilization of ammonium nitrogen by the organisms (17, 18). The substance is described (16) as a non-volatile acid, soluble in chloroform, ether, benzene, and xylene.

The report of Orla-Jensen, Otte & Snog-Kjaer (19) that riboflavin is required by lactic acid bacteria has been confirmed for certain of these organisms by Snell, Strong & Peterson (20) and by Wood, Anderson & Werkman (21, 22). It is denied by Wood, *et al.* (21) for certain other lactic acid bacteria, e.g., *Lactobacillus pentoceticus*. The latter workers showed that certain members of the genus *Propionibacterium* also require riboflavin in addition to the ether-soluble factor, mentioned above, and vitamin B₁. Snell, Tatum & Peterson

(23) reported two factors necessary for the growth of *Lactobacillus delbrückii* on a hydrolyzed casein medium; this report was confirmed by Wood, *et al.* (21), who also added riboflavin to the medium. In a later report Snell, Strong & Peterson (20) described an acidic, ether-extractable substance necessary for the growth and acid production of thirteen species of lactic acid bacteria; the stimulatory effect could be detected in dilutions as high as 1:300,000,000 (0.003 $\mu\text{g. per cc.}$).

Thorne & Walker (24) and Clark (25) have confirmed the claim of Allison & Hoover (26) that an extract of plants, yeast, *Azotobacter*, or commercial cane sugar, added to certain media of purified materials, stimulates the development of the root-nodule bacteria. Thorne & Walker (27) concluded that the necessity of the accessory substance is restricted to synthetic media of unfavorable composition, especially with respect to oxidation-reduction relations. They state that in more favorable synthetic media there is no evidence that an accessory growth factor is essential for either growth or respiration of the root-nodule organism, but certain substances may stimulate the growth of these bacteria.

Weizmann & Davies (28) and Weizmann & Rosenfeld (29) report an "activator" for the acetone-butyl alcohol fermentation which is contained in the dialyzable fraction of yeast autolysate. Using an activator preparation from yeast, they were able to effect a normal acetone-butyl alcohol fermentation in an otherwise synthetic medium.

Saunders & Koser (30) and Saunders, Finkle, Sternfeld & Koser (31) describe the preparation and properties of a liver concentrate which, when added to a synthetic medium, brings about the growth of *Shigella dysenteriae*, *Staphylococcus albus*, and certain other pathogens. Hosoya, Kuwashima, Kayo, Oda & Kagabe (32) have isolated the barium salt of an unknown compound or compounds from a fish extract, for which they report 0.0002 $\mu\text{g. per 5 cc. of medium}$ as the minimum effective dose in producing growth of *Staph. aureus* in an alkali-hydrolyzed peptone or gelatin medium with added amino acids; for *Bacillus botulinus* on the same medium the minimum effective dose was 0.000005 $\mu\text{g. per 10 cc.}$ Details of their tests are lacking, and in view of Knight's findings this report is difficult to evaluate.

Amino acid requirements.—Little work has appeared on this subject during the year. A review by Burrows (33) is of value. Gladstone (9) has investigated the amino acid requirements of 25 strains of *Staph. aureus*. Elaborate precautions were taken to insure

the purity of all amino acids. The amino acids which appeared most necessary for rapid growth were cystine, leucine, valine, proline, glycine, aspartic acid, phenylalanine, and arginine. Histidine and lysine were less important, tyrosine was not required. Most strains grew more slowly in the absence of any one of the more important acids with the exception of cystine, without which no strain continued to grow on repeated transfer. The attainment of absolute results was made difficult by the ease with which the organisms could be "trained" to dispense with certain amino acids. Thus, of the 25 strains tested only four were unable to grow without tryptophane, although thirteen strains were slowed down materially by its absence. Strains giving delayed growth, when subcultured into the tryptophane-free medium, grew without delay. By a process of training, strains of the organism were obtained which were capable of growing in a medium from which all amino acids with the exception of cystine (or an organic source of sulfur) were excluded and in which the main source of nitrogen was ammonia.

Fildes & Richardson (34) showed that for the growth of certain *Staphylococci* any organic sulfur compound containing actually or potentially a mercapto ($-\text{SH}$) group could replace cystine. Since cystine was effective in very much lower concentration than any of the others tested, they concluded that the effect on growth of a mercapto compound was exerted only by virtue of its accessibility as a precursor in the synthesis of cystine.

SYNTHETIC CONSTITUENTS OF THE CULTURE

Lipids.—Reeves & Anderson (35) found that wax formed more than 70 per cent of the total lipids and more than 10 per cent of the dried avian tubercle bacilli. It melted at about 55° , was dextrorotatory, and contained only a trace of phosphorus. On saponification, trehalose, *d*-eicosanol-2 ($\text{C}_{20}\text{H}_{42}\text{O}$), and *d*-octadecanol-2 ($\text{C}_{18}\text{H}_{38}\text{O}$) were isolated and identified. Hydroxy acids of high molecular weight (500 and 1300) were separated but could not be identified. Neither glycerol nor the usual fatty acids were found among the saponification products.

Continuing their work on the occurrence of phthiocerol [$\text{C}_{34}\text{H}_{67}(\text{OH})_2\text{OCH}_3$ or $\text{C}_{35}\text{H}_{69}(\text{OH})_2\text{OCH}_3$], Reeves & Anderson (36) have examined the wax fractions from four new strains of the human tubercle bacillus and found this alcohol present in all the strains. It was also contained in the bovine species (37) but not in the avian organism (38). A colorimetric method for the determina-

tion of the pigment, phthiocol, was developed by Reeves & Anderson (39). The pigment was found in all wax fractions and amounted to from 4 to 16 per cent of the wax. The oxidation of phthiocol in two steps was reported by Hill (40).

Anderson, Reeves & Stodola (41), investigating those lipids of the human tubercle bacillus that are not extracted by neutral solvents, removed these "firmly-bound" lipids from the bacterial residue by extraction with alcohol-ether acidified with hydrochloric acid and, in this way, obtained from 11 to 17 per cent additional lipids. These consisted principally of an unidentified hydroxy acid previously found in the "wax" of the tubercle bacillus, and of a serologically active polysaccharide. Small amounts of lower fatty acids, including tuberculostearic, were isolated. The polysaccharide, which had antigenic properties, gave, on hydrolysis, 57 per cent reducing sugar calculated as glucose. Mannose, *d*-arabinose, *d*-galactose, and inositol were isolated from the hydrolysis products. The unaccounted-for non-reducing constituents of the polysaccharide were assumed to be chiefly a polyhydric alcohol, but no crystalline product could be obtained. The authors were undecided as to whether the lipid-polysaccharide complex existed as such in the cells or was formed during the process of extraction.

From a small quantity of the so-called wax fraction of tubercle lipids, Bloch (42) separated a phosphatide, a carbohydrate, and a wax but did not characterize the constituents of the three substances. The phosphatide was serologically active and contained magnesium but no nitrogen. On removal of the magnesium with hydrochloric acid, the phosphatide titrated as a diglyceride of phosphoric acid. Macheboeuf, Levy & Faure (43) found two serologically active phosphatides in the tubercle bacillus, present mainly as the magnesium salt but also containing calcium and sodium. If the metals were removed progressively, the serological activity disappeared. A unique feature of one of these phosphatides was the presence of inositol, instead of glycerol, as the alcohol part of the molecule.

Anderson, Reeves & Crowder (44) have continued the investigation of the acetone-soluble fat of the leprosy bacillus and found the crude fat to consist of free fatty acids and fatty acid esters of trehalose. The fatty acids proved to be a very complex mixture of at least fourteen different components: Seven were the ordinary saturated fatty acids ranging from caproic to tetracosanoic; three were dextrorotatory, branched-chain, saturated compounds (apparently of the C_{16} , C_{19} , and C_{22} series), and the remainder consisted of both

liquid and solid unsaturated fatty acids (C_{14} , C_{16} , C_{18} , C_{20} , C_{22} , and apparently C_{24} and C_{26}). α - and β -Leprasol and a third phenolic substance not definitely characterized were also isolated.

Carbohydrates.—Heidelberger & Menzel (45) showed that the polysaccharides of a human strain of tubercle bacillus consisted of a complex mixture of serologically-active and -inactive components. Extraction of the defatted cells with dilute acetic acid and fractionation of the extract with organic solvents resulted in the separation of many fractions with varying specific rotations, neutral equivalents, and percentages of ash, nitrogen, and phosphorus. The authors reported evidence for the presence of one serologically-inactive and two -active polysaccharides. One of the latter was insoluble in strong acetic acid, was highly dextrorotatory, and appeared to contain magnesium palmitate and small amounts of pentoses; the other was soluble in strong acetic acid, was weakly dextrorotatory, and contained a relatively high content of pentoses. *d*-Arabinose and *d*-mannose were isolated from the hydrolysis products of both polysaccharides.

Kendall, Heidelberger & Dawson (46) isolated a serologically inactive polysaccharide from cultures of three mucoid strains of hemolytic streptococci. The polysaccharide was acid (neutral equivalent about 380), levorotatory, and contained about 3.7 per cent nitrogen, 11 per cent acetyl groups, and 42 per cent uronic acid anhydride. Reducing sugar, after hydrolysis, accounted for about 80 per cent of the polysaccharide. Glucosamine was isolated from the hydrolysis products, but the uronic acid could not be isolated. The analytical data agree well with those required for a polysaccharide consisting of equal numbers of units of N-acetyl glucosamine and glucuronic acid. The polysaccharide appeared to be identical with that isolated by Meyer, Dubos & Smyth (47) from bovine vitreous humor and from human umbilical cord. Other than glycogen, this is believed to be the only known polysaccharide elaborated both by a micro-organism and by the animal body.

Hassid & Chandler (48) report the isolation of a polysaccharide synthesized by a soil micro-organism. The organism was grown in a mannitol-salts medium without added nitrogen. Although it does not fix nitrogen, it can grow in the above medium with only the impurities in the salts and the distilled water as sources of nitrogen. From this medium, 1 gm. of purified polysaccharide was obtained per liter of culture. It was free of nitrogen, dextrorotatory, and contained only 0.8 per cent ash. On hydrolysis, reducing sugar equiva-

lent to 96.5 per cent of the theoretical for glucose was obtained. The sugar was identified as glucose. Tests for mannose, fructose, galactose, and pentose were negative. Acetylation of the polysaccharide gave a triacetate; iodine titration gave a value nearly four times that of starch; molecular weight determinations by two independent methods gave figures of about 2800. A chain of nine or ten anhydroglucose units is postulated for the polysaccharide.

Other polysaccharides less well characterized chemically have been reported for the following types of bacteria: *Brucella* (49), typhoid (50), paratyphoid (51), *Pneumococcus* (52), dysentery (53), cholera (54), and *Amylobacter* (55).

Proteins.—Pappenheimer and associates (56, 57, 58, 59) published several papers on conditions affecting the production and the nature of diphtheria toxin. They developed a medium of which nearly all the constituents are known and in which a high concentration of toxin is produced. The medium consists of gelatin hydrolysate, methionine, cystine, tryptophane, sodium lactate, maltose, glucose, salts, and a small amount of liver or yeast extract as growth factor. For the production of toxin the iron content was especially important, the optimum being 0.05 mg. iron per liter. Below this figure, less toxin was formed; also, with 0.5 mg. iron per liter no toxin whatever was produced. As this medium gives no precipitate with ammonium sulfate, it is well-suited for the isolation of bacterial products uncontaminated by constituents of the medium. The toxin was obtained from the cell-free filtrate (Berkefeld) by precipitation with ammonium sulfate, and was purified by repeated solution and fractional precipitation with ammonium sulfate, treatment with alumina cream, and dialysis. When maximum potency was reached, the toxin solution was frozen and dried from the frozen state. The white powder thus obtained had the properties of a protein. It contained 16 per cent nitrogen, 0.75 per cent sulfur, no phosphorus, 1.4 per cent ash, 9.0 per cent tyrosine, and 1.4 per cent tryptophane, and gave eight protein color tests, a weak Molisch test, and a negative nitroprusside test. It was precipitated by trichloroacetic acid, and by half-saturated ammonium sulfate; it was heat coagulable, and was unstable in acid solution below pH 6.0. It had a specific rotation of -40 , an isoelectric point of 4.1, and a molecular weight of about 17,000. The lethal dose for a 250 gm. guinea pig was 0.1 μ g.

By a different technique, Boivin (60) obtained a toxin preparation of high potency from diphtheria cultures and showed that the product was essentially protein in character. Purification was effected by

repeated precipitation with trichloroacetic acid at low temperatures and pH 4, decolorization with carbon, and drying of the final precipitate in a vacuum at low temperature. The colorless powder thus obtained had approximately the composition and many of the properties of Pappenheimer's preparation (56).

Mustafa (61) and Bordet (62) have shown that addition of crystalline vitamin B₁ or yeast extract markedly increased the production of diphtheria toxin in the culture.

Northrop (63) has isolated a nucleoprotein from lysed staphylococci which has the properties of bacteriophage, i.e., increases when inoculated into cultures of staphylococci and dissolves the bacteria. Its solubility is practically independent of the quantity in the solid phase (indicating the purity of the preparation); it is digested by chymotrypsin to form an insoluble inactive protein. In diffusion and ultracentrifugal measurements it exhibits a molecular weight of about 200,000,000.

Pigments.—A greenish fluorescent pigment was obtained by Turfitt (64) from cultures of *B. pyocyaneus*, *B. fluorescens liquefaciens*, and *B. fluorescens non-liquefaciens* by adsorption on charcoal, electro-dialysis, and elution with acetic acid. On removal of the acid, the pigment was obtained as a greenish-brown amorphous powder. The yield was 0.8 gm. per 32 liters of medium. The pigment was readily soluble in water, acetic acid, and phenol, but was insoluble in other organic solvents. It was stable to refluxing with zinc and hydrochloric acid, gave off ammonia on dry distillation with zinc dust, was strongly positive to Millon's reagent, and contained 13.3 per cent nitrogen. The absorption spectrum showed a well-defined band at 410 mμ in alkaline solution and one at 370 mμ in acid solution. The pigments obtained from the above three stock cultures and from four new isolations were apparently identical.

On the basis of spectrographic data, Paic (65) believes that the pigment elaborated by the diphtheria bacillus is a copper coprohemo-chromogen, probably having a purine as the base.

The structure of bacteriochlorophyll, the chlorophyll-like pigment of *Thiocystis violacea*, has been further investigated by Fischer & Lambrecht (66). The pigment shows a close similarity to chlorophyll-*a*, and seems to differ from it only in the substitution of an acetyl for a vinyl group on carbon 2, and in containing two more hydrogen atoms.

Toxoflavin (C₆H₆N₄O₂), a yellow pigment isolated from *Bact. cocovenenans*, is a violent heart poison and causes food poisoning

among the natives of Java. It gives a positive murexide test, oxidizes to N-methyl alloxan, and reacts with *o*-phenylenediamine hydrochloride to form N-methyl alloxazine. From these and other data Van Veen & Baars (67) conclude that toxoflavin is isomeric with methylxanthine but possesses a different distribution of double bonds.

BACTERIAL CARBOHYDRATE FERMENTATIONS

It is becoming increasingly probable that the preliminary phases of bacterial carbohydrate dissimilation are very similar to the initial glycolysis stages of yeast and muscle. Although thus far it has been impossible to prepare a cell-free bacterial extract capable of sugar fermentation, much has been accomplished with living cultures, resting cells, and isolated enzymes. Phosphoglyceric acid has been shown by Stone & Werkman (68) to be formed from glucose by cells of nine bacterial genera. It is interesting to note that the compound was not formed by the three *Clostridia* tried. Since pyruvic acid is attacked by most carbohydrate-fermenting organisms, most current bacterial sugar-dismutation schemes give a central position to phosphoglyceric and pyruvic acids.

Butyric fermentations.—Lactic acid, previously considered to be absent from all butyric fermentations, seems to be definitely established as a product, if not as a normal intermediate, of *Clostridium* fermentations. Osburn, Brown & Werkman (69) found that if the pH of a *Cl. butylicum* fermentation is maintained at 7 by addition of sodium bicarbonate, large quantities of pyruvic and lactic acids accumulate. The same authors (70) had previously found that washed cells of the organism readily ferment pyruvic acid to normal products. As cell suspensions did not ferment lactic acid, it seems probable that the lactic acid was formed in the neutral fermentation as an abnormal reduction product of pyruvic acid. Langlykke, Peterson & Fred (71) found that a part of the pyruvic acid added to a growing culture of the same organism appeared at the end of the fermentation as lactic acid. Since lactic acid, as well as pyruvic acid, was fermented in the presence of glucose, the amount of lactic acid found at the end of the fermentation was small. Compounds whose reduction products are not further fermentable, such as acetone and acetylmethylcarbinol, were easily reduced by the culture, and the reduction products recovered at the end of the fermentation. Sjolander (72) reported fermentation balances for a thermophilic *Clostridium*. Here lactic acid seemed to be a normal fermentation product. The organism produced no volatile neutral products.

If pyruvic acid is a normal intermediate in butyric fermentations,

it might be further changed to formic and acetic acids. That such is not the case is indicated by the inability of the organisms to decompose formic acid (70). On the other hand, pyruvic acid might be decarboxylated to acetaldehyde. Repeated failure to isolate acetaldehyde from fermentations containing sulfite (73), together with the fact that acetaldehyde added to a growing culture is largely reduced to ethyl alcohol, makes this seem improbable. Janke & Siedler (74), however, found that cell suspensions of the butyl alcohol organism fermented acetaldehyde, most of which underwent dismutation to ethyl alcohol and acetic acid, although appreciable amounts of butyric acid and butyl alcohol were also formed. Aldol, which is toxic to growing cultures, was fermented by the cells largely to acetic acid and ethyl alcohol, together with a small amount of butyric acid. The poor fermentation balances obtained by these authors, however, make conclusions from their data difficult. The production of acetoin by many *Clostridia* is additional evidence for the intermediary character of acetaldehyde. The effect of various factors on acetoin production has been studied by Yamasaki & Karasima (75).

Severson (76) finds, in agreement with previous workers, that acetic acid added to an acetone-butyl alcohol fermentation is transformed into acetone. Although butyric acid was largely reduced to butanol, some increase in acetone production was also noted on addition of this acid. Langlykke *et al.* (71) have discussed this effect of added hydrogen acceptors. Simon & Weizmann (77) have found that while propionic and butyric acids are metabolized in the acetone-butanol fermentation, dicarboxylic acids are not. Bekhtereva & Ierusalimsky (78) have concluded that the solvent-forming phase of this fermentation is due to onset of a more active decomposition of formic acid, with use of the resulting hydrogen for reduction reactions. However, their data do not show the accumulation of formic acid during the preliminary phase, nor its utilization during the solvent-forming phase.

Underkofler, Fulmer & Rayman (79) have shown that the pentoses resulting from a mild acid hydrolysis of oat hulls may be utilized in the presence of corn mash by the acetone-butyl alcohol organism.

Propionic fermentation.—Wood, Stone & Werkman (80) have formulated a provisional mechanism for the propionic fermentation. Cells of *Priopionibacterium arabinosum* and *P. pentosaceum*, in the presence of toluene, fluoride, and a suitable hydrogen acceptor, transformed glucose and inorganic phosphate into phosphoglyceric acid. Of the acceptors tried, pyruvic acid gave the best yields. The authors

conclude that the primary phases in the propionic fermentation are similar to those in alcoholic fermentation and muscle glycolysis. Since lactic acid has often been shown to accumulate temporarily during the propionic fermentation, it is assumed to be reduced to propionic acid. Since pyruvic acid may be isolated from a propionic fermentation by fixation with sulfite, it is fairly certain that this compound is an intermediate. Moreover, in another paper, Wood, Erb & Werkman (81) show that pyruvic acid is rapidly transformed by *Propionibacterium* cells into acetic acid, carbon dioxide, and propionic acid. It thus appears likely that the intermediary pyruvic acid may be either oxidized to acetic acid or converted by reductive processes to propionic acid. Since in some propionic fermentations, acetic and succinic acids accumulate during the middle stages of the fermentation, and disappear toward the end, both of these compounds must be dissimilated. The authors assume that acetic acid condenses to succinic acid, which then undergoes decarboxylation to form propionic acid. The latter compound, therefore, is formed in two ways. These workers also believe that methylglyoxal may function as an intermediate. In another paper (82) it is shown that concentrations of sodium fluoride which completely inhibit the fermentation of phosphoglyceric acid hardly influence the fermentation of glucose. Fermentation of hexose diphosphate and α -glycerophosphate is also inhibited by fluoride. The conclusion is drawn that at least a part of the normal sugar fermentation does not proceed through phosphorylated compounds.

Fromageot & Chaix (83) have shown that the anaërobic glycolysis mechanism of *Propionibacterium* cells is inhibited by traces of oxygen, but that this inhibition may be prevented by any one of a number of reducing agents. It is also prevented by the addition of a substance extractable from the cells themselves. Therefore, unless small numbers of cells are used, no inhibition is apparent. The authors believe that the inhibition phenomenon is connected with the often observed diminution of anaërobic respiration in the presence of aërobic respiration.

Colon-aërogenes fermentations.—Stone & Werkman (84) report that cells of both *Escherichia* and *Aërobacter* yield phosphoglyceric acid when incubated with glucose and phosphate in the presence of toluene, fluoride, and acetaldehyde, indicating that the preliminary phases of this fermentation also may be identical with those of the alcoholic fermentation. Some of the characteristics of the later stages of the *Aërobacter* fermentation have been investigated by Reynolds & Werkman (85) and by Reynolds, Jacobsson & Werkman (86).

They find that acetic acid accumulates during the initial stages of the fermentation, but later disappears. Added acetic acid is transformed into 2,3-butylene glycol. If sufficient acetic acid is added, the hydrogen production is reduced to zero. Molecular hydrogen, however, is not used to reduce acetic acid. Added succinic acid is also utilized. On the basis of these data and the previously reported fixation of pyruvic acid and acetaldehyde, a provisional mechanism is proposed. The preliminary stages are similar to those of the yeast fermentation. The pyruvic acid formed may be decarboxylated, reduced to lactic acid, or split to form acetic and formic acids, the acetic acid being subsequently reduced to acetaldehyde. Acetaldehyde may be reduced to ethyl alcohol, or be condensed to form acetoin, which is then reduced to 2,3-butylene glycol. A part of the formic acid is decomposed to form carbon dioxide and hydrogen.

Reynolds & Werkman (87) found that the *E. coli* fermentation of xylose gives the same products as glucose fermentation by the same organism. The ratio in which the products occur indicates that the pentose molecule is initially split to a two-carbon and a three-carbon fragment. *A. indologenes*, which produces no succinic acid from glucose, formed considerable amounts of this compound from xylose. Reynolds & Werkman (88) have shown that *E. coli*, normally not a producer of acetoin, forms this compound if subjected to aëration during fermentation. Stahly (89) has found that acetic acid, added to an *Aërobacillus polymyxa* fermentation, is converted largely to 2,3-butylene glycol. Added acetaldehyde is converted to ethyl alcohol, acetoin, and 2,3-butylene glycol.

Lactic fermentations.—Bolcato (90, 91, 92) has studied the mannitol fermentation. His fermentation scheme is unorthodox in that it postulates the formation of only one molecule of mannitol per molecule of acetic acid. He assumes that the hydrogen donated by acetaldehyde to form acetic acid is accepted by unknown constituents of the cells and medium. Bolcato (93) also finds that the mechanism proposed by Nelson & Werkman (94) for the heterofermentative lactic acid bacteria is not applicable to the mannitol-forming lactic acid bacteria studied by him. These organisms do not attack lactic acid, which in the Werkman scheme is the sole precursor of acetic acid. Schoen & Eras (95) have also studied the mannitol fermentation. They find that the pH of the medium has a great effect on the type of fermentation obtained. In the invert sugar fermentation, high pH favors production of mannitol and acetic acid. In the glucose fermentation, high pH favors alcohol production.

Kopeloff & Kopeloff (96) have investigated the forms of lactic acid produced by rough and smooth strains of *Lactobacilli*. The S strains of both *L. acidophilus* and *L. bulgaricus* produced *d*-lactic acid. The R strain of *L. acidophilus* produced *dl*-lactic acid; the R strain of *L. bulgaricus* formed largely *dl*-lactic acid, although slightly more of the dextro form was produced. This work suggests a possible explanation for some of the data in the earlier literature on the effect of environmental factors on the form of lactic acid produced by various cultures.

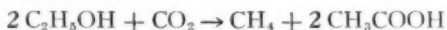
Oxidative fermentations.—Wells, Stubbs, Lockwood & Roe (97) have developed a large-scale method for the production of sorbose from sorbitol, which is now produced industrially from glucose. By application of the pressure aëration method, previously worked out for mold fermentation, to *Acetobacter suboxydans*, it was possible to obtain 93 per cent yields of sorbose from a 15 per cent sorbitol solution in twenty-four hours. This organism has also been used by Underkofler & Fulmer (98) for the preparation of dihydroxyacetone from glycerol. Six per cent glycerol solution gave 90 per cent yields of dihydroxyacetone. Eighty per cent of the product was obtained in pure crystalline form. Utkin (99) has described a new *Acetobacter* species which oxidizes glucose to gluconic and 5-ketogluconic acids. 2-Ketogluconic and *l*-guluronic acids are also reported as present but not definitely characterized. The organism also oxidizes sorbitol, glycerol, and ethyl and butyl alcohols. Dratvina (100) studied the oxidation of acetic acid by *Acetobacter*, and concluded that all species of this genus probably oxidize acetic acid if the proper pH is maintained.

The yield of gluconic acid produced from glucose by *Bact. gluconicum* has been found by Hermann & Neuschul (101) to be almost quantitative in thirty days, at glucose concentrations of 20 per cent and below.

When "resting" cells of oxidative organisms are allowed to oxidize a substrate, theoretical oxygen uptakes are not always obtained, since a part of the substrate is assimilated. Clifton (102) has found, in the oxidation of acetic or butyric acids by *Ps. calco-acetica*, that the addition of such substances as iodoacetate and 2,4-dinitrophenol inhibited assimilation much more than respiration, thus allowing the observance of oxygen uptakes which more nearly approached the theoretical; *B. coli* and *Sp. serpens* gave similar results.

Other bacterial fermentations.—Kluyver & Schnellen (103) have investigated the products formed by *Bacillus rhamnosifermentans*.

This organism, which is remarkable in that it ferments rhamnose more rapidly than glucose, converts the methyl-bearing half of the rhamnose molecule into propylene glycol. Other products are carbon dioxide, hydrogen, formic, acetic, and succinic acids, and ethyl alcohol. Barker (104) has obtained strong evidence in support of van Niel's contention that in the methane fermentation, methane is produced by reduction of carbon dioxide. Barker finds that methane bacteria ferment ethyl alcohol, in the presence of calcium carbonate, according to the equation:



The amount of carbonate used is exactly equal to the amount of methane formed. Since the ethyl alcohol is obviously merely oxidized to acetic acid, the methane must have been produced by reduction of carbon dioxide. If the fermentation is allowed to proceed, the acetic acid is further oxidized to carbon dioxide with the production of more methane, but here, of course, it is impossible to demonstrate that the methane was produced by reduction of carbon dioxide. Conner, Riker & Peterson (105) describe the products of glucose fermentation by the plant pathogens causing crown gall and hairy root. Acetic and pyruvic acids, carbon dioxide, and a polysaccharide gum are produced, but these products account for only 20 to 30 per cent of the fermented sugar. Other metabolites were isolated and partially characterized.

Olson, Peterson & Sherrard (106) have studied the thermophilic fermentation of lignin-containing cellulose. Although added lignin has no effect on the rate of cellulose fermentation, lignin-containing celluloses are not fermented. In order to obtain good fermentation, the lignin content must be reduced to 1 per cent or less. Since extremely fine grinding did not increase the fermentability of lignocelluloses, it was concluded that chemical combination between lignin and cellulose was responsible for the protective action of lignin.

BACTERIAL ENZYMES

Respiration enzymes.—Recent work on the respiration-enzyme systems of bacteria has shown the fundamental reactions of bacterial respiration to be very similar to those in the animal body or the yeast cell. The same coenzymes are active, and the same hydrogen-transfer reactions occur. The lactic dehydrogenase of *B. coli* has been shown by Yudkin (107) to require cozymase, as do animal and yeast lactic dehydrogenases. Lipman (108) has found that the pyruvic dehydro-

genase of *L. delbrückii* requires the presence of cocarboxylase, which is an important coenzyme of yeast and animal carbohydrate metabolism. The dismutation of pyruvic acid to lactic, acetic, and carbonic acids, which is brought about by *Gonococcus* and various *Staphylococci*, has been studied by Krebs (109). Here the reaction evidently involves a simple coupling of lactic dehydrogenase with pyruvic dehydrogenase. A strong activation of the dismutation was brought about by an unknown constituent of boiled yeast juice. On the basis of the later work of Lipman, this activator seems to have been cocarboxylase. Krebs (110) has also shown that the respiration of *Bact. coli commune*, like that of tissue, is catalyzed by fumarate. The anaërobic and aërobic fermentation of glucose, malate, lactate, acetate, and a number of other substrates is greatly stimulated by the presence of fumarate. The fumarate acts as a hydrogen acceptor in the anaërobic experiments; in the aërobic experiments, where succinic acid is rapidly oxidized, the fumarate has the function of a hydrogen carrier. For example, acetic acid, which is not attacked anaërobically, is oxidized to carbon dioxide in the presence of fumarate, the oxidation of one molecule of acetic acid requiring the reduction to succinate of four molecules of fumarate. Aërobically, the succinate does not accumulate, but is reoxidized to fumarate.

Michaelis (111) has noted that the addition of ammonium sulfate to the system succinate-methylene blue-*B. coli* cells will greatly increase the apparent reaction velocity, because of removal of formed fumarate by the fumarase-aspartase system. Pereira-Forjaz, Jacobsohn & Tapadinhas (112) give values for the fumarase- and aspartase-equilibrium constants, which they show to be unaffected by the presence of heavy water. Bach & Lambert (113, 114) have studied the effect of a large number of inhibitors on dehydrogenases of *Staphylococcus aureus*. Wooldridge & Glass (115) report that while the formic, lactic, and succinic dehydrogenases of *B. coli* are still active in cells no longer viable, the activity of glucose dehydrogenase and the amino acid dehydrogenases is greatly reduced in dead cells. The amount of dehydrogenase activity per cell is greatest during the period of logarithmic growth. Yudkin (116) has shown that freezing and thawing of *B. coli* cells destroys the glucose dehydrogenase, but leaves the lactic dehydrogenase unaffected. Both enzymes are destroyed, however, when *Micrococcus lysodeikticus* is lysed by dilute egg white. Stephenson & Gale (117) found that *B. coli* became adapted to galactose fermentation in the presence of galactose only in proportion to the degree of cell multiplication that took place.

Katagiri & Kitahara (118) have continued their studies on biological racemization of lactic acid. The racemizing enzyme is present in cells of the acetone-butyl alcohol organism, in certain *dl*-lactic acid-forming bacteria, and in *Staph. ureae*. It was absent from 29 other bacteria, yeasts, and molds. The interesting observation is also made that two strains of *Lactobacillus saké*, which produce *dl*-lactic acid from glucose, produce only the *d*-lactic acid from arabinose.

By the use of media containing creatinine, Dubos & Miller (119) have isolated bacteria capable of rapid enzymatic destruction of creatinine. Since destruction takes place only in the presence of air, it is evident that an oxidative system is involved. Each molecule of creatinine yields a molecule of urea, which may easily be quantitatively determined. Since other compounds giving a positive Jaffe test for creatinine are not attacked, use of the organisms is considered a valuable tool in creatinine determination.

Proteolytic enzymes.—The secretion of proteinase by living bacteria has received much recent attention. Gorbach & Pirch (120) maintain that only dead cells of *B. fluorescens* secrete proteinase, since, in their experiments, culture filtrates were enzymatically active only to the degree that the culture contained dead cells. They obtained digestion of gelatin but not of casein by culture filtrates. Virtanen & Suolahti (121, 122), however, found that in fifteen-hour cultures of the organism, all of the proteinase was to be found in the medium, and none in the cells. They obtained ready digestion of both casein and gelatin. Weil & Kocholaty (123) have concluded that in cultures of *Cl. histolyticum*, proteinase is secreted by living cells. They find in this organism, as well as in *Cl. sporogenes* and *Cl. welchii*, a proteinase whose pH optimum is 7.0, which is activated by sulfhydryl compounds and by Fe^{++} , Co^{++} , Mn^{++} , Ni^{++} , and Cu^{++} ions. Maximum activation was obtained under anaerobic conditions with a combination of sulfhydryl compound and ferrous ions. Maschmann (124), however, has found that the proteinases of *B. prodigiosus*, *B. pyocyaneus*, and *B. fluorescens*, which also are most active at pH 7, are not activated by sulfhydryl compounds. Gorbach (125) has found that for digestion of protein by filtrates from *Caseicoccus* and *Enterococcus* there are two pH optima, 5.5 and 7.0. Purified *Gastrococcus* and *Caseicoccus* proteinases were slightly activated by hydrogen cyanide at pH 4.7, but inhibited at pH 6.0. For peptidases from culture filtrates and cell autolysates of *Caseicoccus* and *Enterococcus* there were pH optima at both 4.8 and 7.0 on leucylglycine (*Gastrococcus* 4.8 and 8.4). On leucyldiglycine, *Caseicoccus*-enzyme prep-

arations gave pH optima at 4.8 and 7.0. Since all previously studied peptidases have hydrolyzed these substrates most rapidly at a neutral or slightly alkaline reaction, these data are believed to imply the presence in the bacteria studied of a new type of peptidase, active in acid media.

NITROGEN METABOLISM

Amino acid utilization.—Rippel & Lehmann (126) isolated from the intestine of cattle a new spore-forming bacterium (*B. glycino-philus*) which converted 0.1 per cent glycine almost quantitatively into protein in forty-eight hours. Besides glycine the medium contained glucose, salts, and a small amount of agar, acting as a colloid. Iron favored and calcium inhibited the activity of the organism.

Ehrismann & Dramburg (127) found that in the presence of small amounts of peptone, several individual amino acids could not serve as sources of nitrogen for the growth of *Streptococcus pyrogenes*. Furthermore, deamination (as measured by ammonia production) proceeded to only a small extent. Conner, Peterson & Riker (128) found that peptide and amino nitrogen were the chief forms utilized by the crown gall (*Phytoplasma tumefaciens*) organism in the production of protein. When ammonia nitrogen was added to the glucose-yeast-water medium, it furnished a large part of the nitrogen converted into protein. The related hairy root organism (*Phytoplasma rhizogenes*) was less efficient in building protein. Fromageot & Piret (129) stated that amino acids were utilized much less easily than ammonium acetate by species of the propionic acid bacteria. Woods (130) extended the work of Stickland (131) on the coupled reaction between pairs of amino acids brought about by *Cl. sporogenes*, and found that arginine and ornithine, in addition to the acids named by Stickland, could act as hydrogen acceptors. A certain relationship was found to exist between the structure of the amino acid and its role in the Stickland reaction. For example, the simple alpha amino acids, except glycine, act only as hydrogen donors. A *Clostridium* (*Cl. tetanomorphum*) which does not bring about the Stickland reaction was studied by Woods & Clifton (132). Washed cells of this organism, however, attacked many of the common amino acids with the production of hydrogen, carbon dioxide, ammonia, and volatile acid. The most rapid formation of hydrogen occurred with glutamic and aspartic acids, but neither of these equalled pyruvic acid as a source of hydrogen. That hydrogen is not formed *via* formic acid was shown by the failure of the cells to decompose this acid. Steph-

enson & Gale (133) found that the principal effect of glucose on the oxidative deamination of glycine, alanine, and glutamic acid by *Bacterium coli* was to inhibit the formation of the deaminative enzyme system during growth. This inhibition was not attributable to anaërobic conditions. The deamination of glycine by resting cells of *Bac. mycoides* or *Bact. coli* was found by Janke & Tayenthal (134) to be oxidative rather than hydrolytic, since it proceeded only in the presence of oxygen or of *m*-dinitrobenzene. Furthermore, glyoxylic acid was identified as an intermediate in the reaction.

Biological fixation of nitrogen.—Two reviews (135, 136) have appeared which summarize recent advances in our understanding of nitrogen-fixation processes. Krzemieniewski & Kovats (137) have presented further evidence which emphasizes the relation of iron and molybdenum to nitrogen fixation by *Azotobacter*. Virtanen & Laine (138) expressed the belief that aspartic acid is an intermediate in the fixation of nitrogen by this organism.

Wilson and collaborators studied the characteristics of the enzyme system concerned in symbiotic nitrogen fixation. Wilson (139) found that the fixation of nitrogen is a function of the partial pressure of nitrogen in the atmosphere until a level of about 0.15 atm. is reached; above this value fixation is independent of the partial pressure of nitrogen. Wilson & Fred (140) state that oxygen does not appear to be concerned directly in the fixation reaction but does influence the reaction indirectly. Fixation of free nitrogen responded to changes in the partial pressure of oxygen in the atmosphere in a manner which was identical with assimilation of combined nitrogen.

Virtanen and associates propose that the initial product of nitrogen fixation is hydroxylamine which combines with oxalacetic acid to form the oxime; this, in turn, is reduced to aspartic acid. Virtanen, Hausen & Laine (141, 142) offer as evidence in support of this view the finding of *l*-aspartic acid and β -alanine in the sand substrate in which inoculated pea plants have grown. Virtanen & Laine (143) demonstrated that the β -alanine originated through decarboxylation of the *l*-aspartic acid excreted by the root-nodule bacteria present in the substrate. The possibility that the excreted *l*-aspartic acid is a product of the protein metabolism of the plant and not necessarily concerned with the fixation process is rejected by these authors (144). Orcutt's studies (145) on the nitrogen metabolism of soy beans suggest that a product of fixation or transfer may be contained in the basic non-amino fraction.

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GROWTH REGULATORS IN THE HIGHER PLANTS*

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The meristematic tissues in the tips of stems and roots are built of embryonic cells, which are usually nearly isodiametrical in shape and completely filled with protoplasm. Such cells are able to grow in two different ways.

In the meristematic zone most of the cells retain their embryonic appearance. Growth consists of a harmonious increase in the dimensions of the cell wall and of the included protoplasm. The embryonic growth is associated, therefore, with an absorption and assimilation of food substance. When the cells have reached a certain size they divide and the daughter cells are able to grow on in the same way.

Some embryonic cells, however, grow in another way and elongate very much, most frequently in the longitudinal direction of the organ. In this case the growth is brought about by a great increase in the surface of the cell walls, while volumetric increase is not associated with any new formation of protoplasm, since water is embedded in the cells in the shape of vacuoles. This latter form of growth, which initiates the transformation of embryonic cells into somatic ones, is called cell elongation; it occurs behind the growing-points in stems and roots, in coleoptiles of grasses, in young leaves, etc.

The rates of these growth processes are regulated by certain oligodynamic substances which may be called growth regulators. The group of growth substances regulating cell elongation is called the auxin group, the one regulating the embryonic growth is called the bios group.

THE AUXIN GROUP

In order to show that a biological process is initiated or controlled by a certain substance it must be proved that the substance in question is present at the place where the process occurs, and that, in the concentration in which it appears, it is able to have a regulating effect on the process.

The auxin formed at the tip of the *Avena* coleoptile fulfills these requirements. When the tip is cut off and put on unilaterally, the resulting curvature shows that the quantities of growth substance given

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off by the tip have a regulating effect on the cell elongation in the basal portion of the coleoptile, where it can be demonstrated by the trapping ("diffusion") [Søding (2)] or the extraction method [Thimann (1)]. As is well known, auxin is probably present in all higher plants, where it seems to have the same function as in the *Avena* coleoptile, but it is possible that it may have other functions as well.

QUANTITATIVE METHODS

A quantitative determination of the amount of auxin in a plant organ may be made by carrying it over into a plate of 1.5 per cent agar. Out of this plate blocks are cut and placed unilaterally on decapitated *Avena* coleoptiles; a curvature will then result, the magnitude of which is a function of the amount of auxin in the agar. When, therefore, the magnitude of the curvature is measured and a unit for the auxin is selected, it is possible to make a quantitative determination, expressed in these units.

The growth substance may either be secreted from the plant organ directly into the agar plate or it may be extracted and later on transferred into the agar. When the first method is used, the plant organ, a coleoptile tip, root tip, or stem piece, is placed on a plate or block of agar [Went (1)] or dextrose agar. After some time the plant organ is removed and the auxin content of the block determined. This method measures the quantity of auxin which is given off during a certain time from a center of production or from a plant organ. No information is obtained, however, as to the momentary, absolute concentration of auxin in the plant organ, since the amount obtained depends partly upon whether or not new auxin is formed and likewise upon the rate of its secretion. Under certain conditions the method may be used to determine differences in concentration. In some cases the method suffers from entirely incalculable faults, as in order to enter into the agar the auxin has to pass a cut surface, where a destruction may occur [Thimann (1); Fiedler].

When the second method is used, the growth substance is extracted by means of chloroform [Thimann (1)], alcohol, or ether. A measured amount of the extract is evaporated, the residue is dissolved in ether and is sucked up into a pipette, from which little by little it is dropped onto a plate of agar of 1 sq. cm., where the ether evaporates. The growth substance then passes over into the agar and can be measured [Boysen Jensen (8)]. The extract of auxin must often be purified before the determination. By this method the momentary

content of auxin in a plant organ is measured and, presumably, an expression of the actually effective amount is thereby obtained. The extraction method must be regarded, therefore, as the one best suited for determination of auxin. The possibility of the growth substance being partly localized in certain cell elements or of being present in a latent condition must still be taken into consideration.

If the effect of auxin preparations on plants (as far as possible naturally occurring growth substances and not artificial ones must be used) is to be determined it can be done by mixing the preparation with lanolin (*Adeps lanae*) and spreading this paste on intact coleoptiles, or other plant organs [Laibach (2)]. If one wishes to determine the dependence of growth upon the concentration of auxin, the plant organ in question (coleoptile, root) is placed in such a large amount of solution that the concentration will, to all practical purposes, remain unaltered during the experiment (Jost & Reiss; Geiger-Huber & Burlet). In such a case the effect may be measured by determining the increase in length.

CHEMISTRY OF THE AUXINS¹

Kögl, Erxleben & Haagen Smit have isolated two different auxins from malt and maize oil, namely auxin A, $C_{18}H_{32}O_5$ (auxentriolic acid), and auxin B, $C_{18}H_{30}O_4$ (auxenolonic acid). By means of the diffusion method the molecular weight of the auxin found in the tip of *Avena* and maize coleoptiles was determined as 376 [Went (1)] and 346 [Kögl, Haagen Smit & Erxleben (2)], respectively; these values correspond rather closely with the molecular weight of auxin A, which is 328. The behavior of the growth substance from the tip of the *Avena* coleoptile in acids and alkalis likewise makes it probable that it is identical with auxin A. The auxin regenerated in the decapitated *Avena* coleoptile, as well as the growth substance secreted by the root tip of *Vicia faba*, likewise seem to be identical with auxin A according to molecular weight determinations [Heyn (2)].

Auxins A and B easily become oxidized and thereby lose their activity. A substance which destroys the growth substance is found in the expressed sap of many plants [Thimann (1); Kornmann; van Overbeek (2)]. The substance is thermolabile and is presumably an oxidizing enzyme (Larsen).

¹ The β -indolyl-acetic acid and artificial growth substances have not been proved to be phytohormones in higher plants and shall therefore be discussed later.

From auxin A a lactone easily arises which is transformed by ultraviolet light into a physiologically inactive substance, lumi-auxin-A-lactone (Kögl).

FORMATION OF AUXIN

Auxin is often formed in special centers of production, which in most cases probably consist of embryonic cells. The following examples may be mentioned: coleoptile tips, shoot tips, cotyledons of *Raphanus*, young leaves, developing fruits. The root tip, too, is such a center of production. It was maintained that auxins in the root tip were transported there from the shoot [Went (3); Thimann (1); Fiedler], but it can be demonstrated with certainty that isolated root tips of *Vicia faba* are able to form auxin when supplied with sugar [Boysen Jensen (7); Nagao; Raalte], and there can be very little doubt that such formation of growth substance occurs under natural conditions also.

The formation of auxin in seedlings is generally dependent on a supply of stored food materials. It has been maintained that the growth substance secreted by the tip of the *Avena* coleoptile is not formed in that place but is supplied from the seed, either as such (Pohl) or in an inactive form (precursor) which is transformed into growth substance in the tip (Skoog). Still, it must be remembered that the root tip of *Vicia faba* is itself able to synthesize growth substance and there is some reason to believe that the sugar present is used in this process (Cholodny).

Light is generally supposed to be a necessary condition for the formation of auxin [van Overbeek (1); Thimann & Skoog; Avery]. Perhaps this may be correct as far as the green parts are concerned but it cannot hold true generally. It must be supposed that the formation of auxin in the root tip can occur in darkness.

A unilateral influence of gravity is able to call forth formation of auxin in nodes of grasses which have ceased growing and which do not contain any auxin (Schmitz).

TRANSPORT AND DISTRIBUTION OF AUXIN

From the centers of production in coleoptile tips, stem tips, root tips, and young leaves auxin is transported in the basipetal direction; consequently a stream of growth substance occurs through the growing parts and beyond. The transport of auxin in the concentrations

occurring in the intact plant usually takes place only in the longitudinal direction and is strictly polar. The concentration of growth substance decreases the farther one proceeds from the center of production [Boysen Jensen (3); Thimann (1); Thimann & Skoog; Sjøding (2)]; it is partly used for growth and is partly destroyed in other ways (Bonner & Thimann).

The transport of auxin may occur in parenchymous cells, as in the *Avena* coleoptile. In leaves it occurs also in the veins (Avery), probably in the phloem. The rate of transport in the *Avena* coleoptile is about 10 to 12 mm. per hour (van der Weij). In leaves, where the transport occurs in the phloem, a velocity up to 185 mm. per hour was measured (Laibach & Fischnich).

The great rate at which longitudinal transport of auxin occurs could be supposed to arise through diffusion in connection with protoplasmic streaming. Such processes, however, do not suffice to explain the fact that the transport of auxin is polar and that the auxin can be transported from lower to higher concentrations. Such a transport requires energy. Since the auxins are acids the possibility has been considered that the transport might be brought about by electric potential differences, such that the auxin would travel towards regions of positive charge. But experiments made by Ramshorn, Hellings, and Clark show that there is no direct connection between potential difference and displacement of growth substance. Likewise, an artificial growth substance, the methyl ester of β -indolyl-acetic acid, although not an acid, can be transported longitudinally in the *Avena* coleoptile [Kögl & Kostermans (2)].

Nothing is known about the distribution of auxin within the protoplasm. It is possible that the hydrogen-ion concentration of the protoplasm may affect its distribution. Thus when the protoplasm is alkaline auxin may occur in aqueous solution, but when the protoplasm is acid it may be dissolved in the lipoids of the protoplasm.

PHYSIOLOGICAL EFFECTS OF AUXIN

Cell elongation.—The auxins have, above all, a regulating effect on cell elongation; they may accelerate and also retard this process. The effect on the plant cells is determined partly by the concentration of auxin and partly by the reactivity of the cells to auxin. The reactivity, which may vary with regard to quality as well as quantity, comprises all the factors which influence cell elongation, with the exception of the concentration of growth substance.

It has been shown with certainty that auxin is necessary for the growth of the *Avena* coleoptile and for that of the seedling stem. A supply of growth substance will generally accelerate the rate of growth in these organs. Although the growth of the root is inhibited by a comparable supply of growth substance, still, recent experiments (Jost & Reiss; Amlong; Fiedler; Geiger-Huber & Burlet) seem to indicate that the elongation of roots containing very little auxin (decapitated roots or roots in organ culture) is accelerated by small amounts of β -indolyl-acetic acid. This suggests that auxin is necessary for the growth of the root as well. Hence the growth of all plant organs probably stops if no auxin is present. For all organs low concentrations of auxin have an accelerating effect, whereas high concentrations have a retarding effect; the relation between the rate of growth and the concentration of auxin may therefore be expressed by an optimum curve. The optimal concentration of auxin is, however, much lower for the root than for the stem and coleoptile. As a consequence the concentration of auxin in the root is generally too high, whereas in coleoptiles and stems it is too low to give maximum growth.²

The opposite effect of almost the same concentration of auxin on the growth of roots and stems indicates that the reactivity is not the same in all cells. Even in cells of the same kind the reactivity may vary greatly, as it is influenced partly by internal factors (age, stage of development [du Buy; Went (4)] and partly by external factors (e.g., light).

The effect of auxin is a change in the rate of surface growth of the cell wall. How this occurs is not known. Heyn (1) thinks that the increase in the rate of growth of the *Avena* coleoptile is due to the fact that the plasticity of the cell wall increases, whereas Söding (1) maintains that it arises from an increased new formation of cell-wall substance (intussusception). If Heyn's hypothesis is correct, then the growth-stimulating effect of the auxin in the *Avena* coleoptile really consists in a reduction of the inhibiting effect which the stiffness of the cell wall imposes on the rate of growth.

Growth movements in orthotropic organs.—When the concentration of growth substance and the reactivity are uniform on all sides of a cylindrical plant organ, the rate of growth will likewise be uni-

² Czaja maintains that the inhibiting effect of the growth substance on the growth of the root is due to the fact that two streams of growth substance, opposite in direction, meet in the root. This idea is not acceptable [Thimann (3)].

form. If, on the contrary, a unilateral effect of an external factor gives rise either to an asymmetrical distribution of growth substance or to a different reactivity of the cells on opposite sides of the organ, growth will likewise be different on opposite sides. When the external factor causes an asymmetrical distribution of the growth substance and also alters the reactivity of the cells, these two effects may either strengthen or weaken each other.

The asymmetrical growth which may thus result as the consequence of a unilateral influence of an external factor will cause the plant organ to bend and orient itself in relation to such a factor. Such curvatures are called tropisms. They are divided, according to the factor responsible for the curvature, into geotropisms, phototropisms, etc. When the movement occurs toward the factor in question it is called positive, when in the opposite direction, negative. The tropisms are of essential importance for the orientation of plant organs in space.

Asymmetrical distribution of auxin.—We shall first examine the asymmetrical distribution of auxin and its significance. Among the external factors, light and gravity especially are able to call forth, by a unilateral influence, an asymmetrical distribution of growth substance in orthotropic plant organs. The asymmetrical distribution of the growth substance very easily arises in the centers of production and because of the longitudinal transport of the auxin a corresponding asymmetrical distribution occurs basipetally in the growing parts. Such unilateral distribution of auxin may be shown by means of the trapping method (in coleoptile tips [Went (1); Dolk], in seedling stems [van Overbeek (1); Dijkman; van der Laan], in root tips [Boysen Jensen (4)]) or by the method of extraction (in seedling stems [Boysen Jensen (7)]).

The primary effects of light and gravity are unknown. As the sensitivity to different wave lengths is identical with the absorption spectrum of the carotenoids, Castle supposes that these substances are of importance in the perception of light. The primary effect of gravity is perhaps due to the fact that certain bodies (according to Haberlandt, starch grains) exert a pressure on sensitive protoplasmic elements. Brauner has shown that when stems and roots are placed horizontally a geo-electric potential is formed, the lower side thereby becoming positive.

The asymmetrical distribution of auxin may be due (a) to unilateral retardation or acceleration of longitudinal transport, (b) to

unilateral destruction or formation, (c) to transverse displacement. Moreover, two or more of the above mentioned factors may work together.

The first of the above possibilities is especially dealt with by du Buy & Nuernbergk. However, it has not been possible to show any effect of diffuse illumination on the rate of transport of growth substance in the hypocotyl of *Raphanus* [van Overbeek (1)] or in the *Avena* coleoptile [Boysen Jensen (2)].

The possibility that a unilateral distribution of growth substance may be partly due to the destruction of auxin by light has repeatedly been maintained (Paál; Burkholder & Johnston; Kögl). The effect of light may be direct or may be brought about indirectly by means of an enzyme. But a unilateral destruction of auxin cannot explain the fact that the concentration on the shaded side of a unilaterally illuminated coleoptile becomes greater than normal. Further it is certain that a destruction of auxin by light cannot give rise to the asymmetrical distribution of auxin which occurs as a consequence of geotropic stimulation. Haberlandt thinks that the latter arises out of the fact that the pressure of the starch grains calls forth a unilateral formation of growth substance.

It was shown by Boysen Jensen (1) that a transverse displacement of substance takes place in the tip of the coleoptile under the influence of unilateral light. Displacement of auxin in decapitated coleoptiles has been shown by Dolk and Boysen Jensen (2), in seedling stems by Dijkman and van der Laan. Only part of the growth substance is displaced (Koch). This transverse displacement of the growth substance is the most important cause of the unilateral distribution of growth substance.

Such a displacement of auxin must require energy. Since the auxin is an acid it has been thought that the displacement is due to a potential difference (possibly the above-mentioned geo-electric potential). If an *Avena* coleoptile is placed in an artificially produced electric field the coleoptile will curve slightly toward the positive electrode; it seems to the writer that one must expect a curvature in the opposite direction.

Koch has shown that passage of an electric current through the plant with a tension of 10 to 100 millivolts is able to produce curvatures toward the negative electrode in *Helianthus* hypocotyls.

At the present moment the whole problem of the transverse transport of auxin is very obscure. It will be of importance to examine

whether the methylester of β -indolyl-acetic acid can be displaced transversally.

If it cannot be demonstrated that the displacement of auxin is due to an external gradient of energy it must be postulated that the energy necessary for the displacement arises from the plants themselves, and that the cells are able to transport the growth substance in a direction determined by the external factor.

Cordes & Laibach have shown that the shading of one cotyledon on the seedling of *Cucumis* calls forth a curvature of the hypocotyl toward the illuminated cotyledon. They suppose that the growth of the hypocotyl is dependent partly on auxin, and partly on a *Dunkelstoff*, which is formed in darkness but is destroyed in light or transformed into an inactive form. The portion of the hypocotyl below the shaded cotyledon will therefore grow more than the opposite side. In a unilaterally illuminated hypocotyl there will be more *Dunkelstoff* on the shaded than on the illuminated side and, according to the above-mentioned investigators, this will cause a phototropic curvature, either alone or in connection with a displacement of growth substance.

An unequal distribution of auxin in the growing parts will generally bring about an unequal growth which will result in a curvature, a growth movement. In organs which have a sub-optimal concentration of auxin (coleoptiles and stems) the side which has the largest concentration will grow most rapidly; the phototropic reaction will be positive and the geotropic reaction negative. In organs such as roots, which have super-optimal concentrations of growth substance, the opposite will be the case; the geotropic curvature will be positive. If the auxin content in roots is greatly diminished, the reaction may, according to Amlong, pass from positive to negative.

Reactivity of the cells.—Next we shall treat the alteration of the reactivity of the cells and its significance. Van Overbeek (1) has shown that the growth of a *Raphanus* hypocotyl is inhibited by light. This is due neither to an effect on the transport of growth substance nor to a consumption of growth substance, and must therefore arise out of an alteration in the reactivity. The positive phototropic curvature of the hypocotyl of *Raphanus* is due, therefore, partly to a displacement of auxin, and partly to a greater reduction in the reactivity of the cells on the illuminated than on the shaded side. In later experiments, however, van Overbeek (2) comes to the conclusion that light calls forth a destruction of auxin in the *Avena* coleoptile.

In hypocotyls of *Helianthus* the phototropic curvature perhaps

arises exclusively from the inhibiting effect of light on the reactivity of the cells, according to Blaauw's theory.

Whether the alterations examined by Warner and others [compare Metzner (1) (2)], especially in the hydrogen-ion concentration, on the opposite sides of stems placed horizontally or illuminated unilaterally are of any importance to the reactivity of the cells, and thereby to the geotropic and phototropic curvatures, is not known. It has been suggested that an increase in the hydrogen-ion concentration might be responsible for a liberation of latent auxin (Bonner).

Unsolved problems.—The phototropic and geotropic curvatures of the *Avena* coleoptile may possibly be explained by displacement of auxin alone, and the phototropic curvatures of seedling stems, presumably, by displacement of auxin and alteration of the reactivity of the cells. As far as the geotropic curvatures of seedling stems are concerned, no alteration of the reactivity has been shown. A quantitative examination of the distribution of auxin seems to show that the difference between its concentrations on the upper and lower sides is less than might be expected from the magnitude of the curvature [Boysen Jensen (7)].

It would be of extreme importance if it could be shown that organs which contain no auxin are able to exhibit tropistic movements. This would mean, first, that the importance of the auxin to cell elongation is not general and, second, that there are other tropistic mechanisms than those mentioned above. Faber was not able to prove the existence of auxin in roots of *Agrostemma githago* and *Lepidium sativum*; but it is possible that its presence may be demonstrated by other methods. Roots cultivated in artificial substrate offer greater difficulties. In experiments carried out by Fiedler, roots cultivated in this manner contained no auxin but were still able to curve geotropically. The objection can be raised that the culture medium used by Fiedler probably contained auxin, but other problems still exist. Geiger-Huber & Burlet have found that β -indolyl-acetic acid in very slight doses is able to stimulate the growth of such roots.³ This probably means that the content of auxin is so low that a negative curvature should be expected if the roots are able to react to gravity. The experiments ought to be repeated with auxin.

Another difficult problem is presented by tendrils, in which an acceleration of the mean rate of growth takes place during the thigmo-

³ The culture medium did not contain auxin.

tropic movement. This may be due to an increase of the reactivity of the cells or to a liberation of auxin on the side which is not stimulated.

Growth movements in plagiotropic organs.—The growth of the tips of *Parthenonarcissus* shoots is straight at first. Later, the shoots curve positive-geotropically, and at last become straight again. At all stages more auxin is extant on the upper than on the lower side. The above-mentioned curvatures are due, therefore, to a changing reactivity toward auxin of the cells on the upper and lower sides (Kaupp; Zimmermann).

Other effects.—The function of the auxins in plants is, above all, to regulate cell elongation; in addition they seem to be able, especially in large concentrations, to exercise both exciting and also retarding effects on other cell functions, for which reason they are important as factors correlating the different parts of the organism. It was shown [Snow (2)] that auxin is able to stimulate cambial growth of *Helianthus*. It has been suggested that the auxin formed by the shoots during frondescence initiates the cambial growth of trees in the spring. Whether such an effect is transported from the branch tips all the way down the trunk has not been demonstrated with certainty.

The formation of roots on cuttings is greatly furthered if there is a developing bud on the cutting (van der Lek). The suggestion is therefore obvious that the formation of roots is due to substances given off from the bud. Went (2) and Bouillenne & Went have shown that a root-forming substance (rhizokaline) is present in leaves, germinating barley, etc. Recent experiments (Thimann & Went; Laibach, Müller & Schäfer) have shown that pure auxin is able to bring about a formation of roots. Whether under normal conditions it is auxin alone that gives rise to the formation of roots, or whether it works in connection with other substances (rhizokaline, wound hormones) has not been proved.

As shown by Dostál a leaf will inhibit the development of its bud and a developing bud is responsible for an even greater inhibition. If, further, the terminal bud of a seedling plant of *Phaseolus* or *Vicia faba* is removed the lateral buds will develop. Snow (1) was the first to show that the inhibiting effect of the terminal bud probably is due to a soluble substance. Later it was proved that the development of the lateral buds of *Vicia faba* could be prevented by supplying auxin to the cut surface (Skoog & Thimann). Some investigators [Laibach (1); Le Fanu] think that the inhibiting effect is brought about

indirectly, whereas Thimann (4) maintains that the auxin, in relatively high concentrations, is directly able to inhibit the development of buds.

β -INDOLYL-ACETIC ACID AND ARTIFICIAL GROWTH SUBSTANCES

It was first shown by Niels Nielsen that under certain conditions of culture a growth substance is formed by *Rhizopus suinus* and *Absidia ramosa*, which influences cell elongation in *Avena*. As afterwards appeared, it is also formed by many other micro-organisms when cultivated on peptone, but is not, however, identical with auxin. As shown by Kögl & Kostermans (1) the substance is β -indolyl-acetic acid, which can also be isolated from urine [Kögl, Haagen Smit & Erxleben (1)]. It arises through the decomposition of tryptophane. It has not yet been demonstrated in higher plants, and it seems to be of no importance to the lower organisms in which it is formed.

The physiological effects of β -indolyl-acetic acid on higher plants are about the same as those of auxin, but there are certain differences which may be due to the fact that it does not become oxidized so easily. It accelerates and retards cell elongation in coleoptiles and roots, initiates growth in secondary meristematic tissues, as well as formation of callus and roots, causes inhibition of bud development and may likewise call forth epinastic movements in leaves (for the analysis of such movements see Fischnich).

At the Boyce Thompson Institute, Crocker, Zimmermann, Hitchcock, and Wilcoxon have in recent years shown that 32 different substances in all, especially aromatic acids and their esters, are able to bring about formation of roots, proliferations, intumescences and epinasty—a series of effects similar to those which are also brought about by auxin and β -indolyl-acetic acid (compare Zimmermann & Hitchcock). Some of these substances, e.g., β -3-indole-propionic acid and α -naphthalene-acetic acid, are also able to call forth curvatures in *Avena* (Avery, Burkholder & Creighton).

A special group within these substances is formed by the four gases ethylene, acetylene, propylene, and carbon monoxide, which are likewise able to induce the formation of intumescences and roots. They are generally unable to accelerate cell elongation, but in large doses are able to inhibit this process (Crocker, Hitchcock & Zimmermann). Whether the ability of ethylene to induce root formation is comparable to the effect of auxin is for the present an unsettled question.

It is, then, a strange fact that substances which have nothing in common chemically still produce a series of similar physiological effects. This is best understood by supposing that the regulating influence of growth substances is brought about through their effects on the protoplasm, and that such primary effects are transferred to the cell wall, possibly by various intermediate steps. Even if the primary effects are different, nevertheless the same final effect, possibly an alteration of the cell wall, can result. This is expressed by saying that the growth substances are stimulants (Fitting).

BIOS GROUP

In many micro-organisms there occurs only embryonic growth. It is well known that some of these organisms have lost their ability to grow on purely synthetic substrates. This is especially true in such cases as most pathogens, yeast, and lactic acid bacteria—forms which grow on living organisms, their extracts or their secretions. Wildiers was the first to show that this was the case with ordinary beer yeast, which is only able to develop if, besides sugar, ammonia, and the necessary salts, a substance or complex of substances, which he called bios, is added.

As these substances which influence the growth of yeast are very widely distributed in higher plants it was postulated that they were also of importance to the embryonic growth of the latter (Dagys). Kögl & Haagen Smit examined the effect of biotin (a preparation isolated from egg yolk and greatly influencing the growth of yeast) on pea seedlings, the petioles of which had been removed, and found that it had a slightly accelerating effect on the growth. It was likewise demonstrated that when suitable amounts of auxin and cane sugar were added, biotin increased the number of roots formed on cuttings [Went & Thimann (p. 239)].

Extracts of yeast also seem to play an important part in connection with organ cultures of roots (Robbins; White).

Conclusion.—The harmony which characterizes the growth and development of living organisms is perhaps their most essential feature. This harmony is indispensable to a successful integration of the numerous biological processes which result in the perfectly co-ordinated complex structure. It appears only when the processes of life are mutually regulated. One of the deepest problems of biology is, therefore, the nature of this regulation. It is toward the answer to this question that the investigation of growth substances has made its

contribution, the significance of which should be neither over- nor under-rated. Since it is clear that all development can be ultimately traced to growth processes, those physical and chemical factors which regulate growth will be of fundamental significance for the organism. In plants, growth substances are the most important of these chemical factors yet known, but it is obvious that they are only a tool and that the regulating principle proper is still obscure. Consequently, the investigation of growth substances is only a stage along the road and we must delve still deeper into the problem.

Comprehensive accounts of the investigation of growth substances and their significance are found in the following papers and books cited in the bibliography: Boysen Jensen (5, 6); du Buy & Nuernbergk; von Guttenberg; Jost; Otte; Schlenker; Went & Thimann.

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